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13. ABSTRACT ***(Maximum 200 Words)***
The purpose of this study is to identify novel genes that encode proteins that can be used to detect ovarian cancer before it spreads outside the ovary and becomes incurable. The goal is to assemble a panel of known and novel ovarian tumor markers that may form the basis of a cost-effective, serologic screening test for early stage ovarian tumors. The research encompasses the use of two novel technologies to identify such genes. In Project 1 we use high density array hybridization (HDAH) to identify genes that are over-expressed in ovarian cancer tissue. In Project 2 novel ovarian tumor antigens are being identified by an immunoscreening procedure known as SEREX. Project 1 investigators have identified two potentially interesting genes already, and their hybridization work is not yet completed, so we anticipate having several candidate genes for assay development next year. Project 2 investigators have optimized methods for removal of E.coli-specific antibodies present in serum samples, while maintaining antibodies to known tumor antigens. In addition, they have successfully constructed and performed a primary SEREX screen with an ovarian tumor cDNA library. Finally, they have cloned p53, a known ovarian tumor antigen, along with hZF5, a novel candidate ovarian tumor antigen.

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Nicole Urban 10/29/99

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October 1, 1998 – September 30, 1999
Year 01

Use of Novel Technologies to Identify and Investigate Molecular Markers for Ovarian Cancer Screening and Prevention

Nicole Urban, ScD
Principal Investigator

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INTRODUCTION:

The subject of this research is ovarian-cancer-related gene discovery. Our purpose is to identify novel genes that encode proteins that can potentially be used to detect ovarian cancer before it spreads outside the ovary and becomes incurable. The goal is to assemble a panel of known and novel ovarian tumor markers that may form the basis of a cost-effective, serologic screening test for early stage ovarian tumors. The scope of the research encompasses the use of two novel technologies to identify such genes. In Project 1 we use high density array hybridization (HDAH) to identify genes that are over-expressed in ovarian cancer tissue. In Project 2 novel ovarian tumor antigens are being identified by an immunoscreening procedure known as SEREX. Novel genes identified by HDAH will be evaluated for development as tumor markers. Similarly, antigens identified by SEREX will be further evaluated using purified proteins and larger numbers of normal, benign and cancer serum in an ELISA format. Statistical analyses will be employed to determine the sensitivity and specificity of HDAH- and SEREX-defined tumor markers for the detection of early-stage ovarian cancer. Towards this goal, serum antibody responses to the known tumor antigens p53, HER2 and Myc are being evaluated by ELISA in patients with ovarian cancer versus normal controls.

BODY:

Project 1
Identification of Potential Markers for Population Based Screening for Ovarian Cancer: Characterization of Differential Gene Expression in Malignant Neoplasia by Use of High Density Array Hybridization (HDAH).
Nicole Urban, ScD, Leroy Hood, Ph.D., MD, Michel Schummer, Ph.D., Nancy Kiviat, MD

The goal of this project is to identify potential markers for population based screening for ovarian cancer through characterization of differential gene expression in malignant neoplasia by use of High Density Array Hybridization (HDAH).

Work proposed:

Task 1: Generation of representative cDNA arrays from early and late stage ovarian carcinomas. Six cDNA libraries will be generated from fetal, normal and benign, early stage and late stage neoplastic ovarian tissues. These libraries will then be used to construct first generation solid-phase membrane arrays containing 100,000 clones.

Task 2: Primary characterization of normal and neoplastic tissues using these arrays.
Phase 1: Hybridization of the first generation membranes with cDNA probes derived from 9 normal ovarian tissues, 4 benign cystadenomas, 7 early stage and 7 late stage ovarian serous adenocarcinoma, 2 samples of bone marrow and 2 samples of liver tissue.
Phase 2: Evaluation of hybridization results and selection of 2000-3000 clones overexpressed in malignant ovarian tissues.
Phase 3: Construction of second generation cDNA arrays containing these 2000-3000 clones.

Task 3 (in its initial phase): Further characterization of gene expression in normal and neoplastic ovarian tissue. Hybridization of the second generation arrays with cDNA from tissues used in Task 2 plus 24 additional normal tissues (20 ovarian, 2 bone marrow and 2 liver controls), 15 cystadenomas, 20 additional early and 20 additional late stage ovarian serous adenocarcinoma.

Work accomplished:

Task 1: Generation of representative cDNA arrays from early and late stage ovarian carcinomas

Representative cDNA arrays have been generated, but we had to depend primarily on tissue from women with late-stage ovarian carcinoma because very few early-stage cases have been available for tissue harvesting. As described below in the Core section, no cases of early-stage invasive serous carcinoma have been enrolled in the study to date, despite our best efforts. The design of the HDAH work did not allow for much delay, especially in the initial stages where gene expression data is accrued. An alternative acquisition process of early stage serous carcinomas through the GOG did not result in high quality tissues in a short time. We therefore decided to move forward with the generation of the other libraries and use the early stage tumors only for probe generation.

The foreseeable downside from this was an array that lacks genes whose expression is restricted to those early

stage tissues. Any gene that continues to be expressed in the later stages of carcinogenesis however would still be present even if expressed at low levels According to our calculations, a given gene needs to be expressed at around 10 copies per cell in order to be reflected at least once within the pool of the 20,000 clones that we wanted to select from each library for arraying on membrane. This category of gene expression is commonly regarded as the low category, (Zhang, Zhou et al. 1997) and in it we will find roughly 25% of all genes expressed in a given tissue.

We therefore created four cDNA libraries from pooled tissues (20 pooled fetal ovaries, 4 benign ovarian cystadenomas, 3 normal ovaries, 4 late stage serous ovarian carcinomas) plus an additional library made from 6 metastatic ovarian carcinomas. For quality control, from each library, 96 clones were randomly chosen. The clones were sequenced and analyzed by similarity analysis against the non-redundant and EST database. The criteria for a satisfactory cDNA library were an average insert size around 1 kb, a low number of mitochondrial and ribosomal sequences, a limited number of clones with no insert, and significant cDNA diversity (Nelson 1998) .

	fetal	normal	benign	Late	Metastatic
mean insert length (kb)	6.00000000e-1	9.00000000e-1	7.00000000e-1	1.20000000e+0	1.30000000e+0
titer (transformants/ml)	3800	2400	9700	6300	29300
mitochondrial	6%	4%	5%	4%	3%
Ribosomal protein	7%	2%	5%	4%	2%
Genomic / repeat	18%	7%	19%	6%	6%
no insert	21%	4%	19%	5%	6%
EST	20%	34%	25%	37%	35%
no match	9%	13%	12%	10%	13%
Known genes	19%	36%	15%	34%	35%

Table 1
Result from sequencing 96 clones from each of the 5 cDNA libraries

As shown in Table 1, only three libraries fulfilled these criteria. For the fetal and benign libraries, the average insert sizes were considerately below 1 kb. In addition, the number of clones without insert plus the ones with repeats or genomic fragments exceeded one third, a number far too high for consideration to array. Diversity of clones with homology to known genes was similar in all cases. The titer of the three remaining libraries was small which reflects the fact that we chose not to amplify the libraries in order to have a better representation of the lowly expressed clones. We selected 102,680 clones from the three libraries (9,216 from the normal, 17,664 from the late stage and 83,712 from the metastatic library each) and arrayed the colonies onto 32 sets of 5 nylon membranes, each holding 20,536 colonies. The colonies were lysed and the DNA was fixated onto the membranes [Nelson, in print #633]. One set of membranes was hybridized with a probe recognizing the vector portion of each clone. The resulting hybridization pattern revealed that out of the 120,680 colonies that were arrayed, 97,803 actually grew on the membranes. Appendix A, Figure 1 shows a close view on one such membrane where more than 95% of the colonies give a positive signal with the vector probe.

<u>Task 2: Primary characterization of normal and neoplastic tissues using these arrays</u>

Redefinition of Task 2:

In Task 1 we generated 32 sets of high-density cDNA array membranes consisting of 5 membranes each. We had proposed to hybridize them with first-strand cDNA probes from the following tissues: 9 normal ovaries, 3 skeletal muscle samples, 4 benign cystadenomas, 7 early stage and 7 late stage ovarian serous adenocarcinomas. However, the 7 early-stage serous tumors were not available. We realized that, at best, we would accumulate these early stage tissues by the end of 1999. This was, according to protocol, shortly after Task 3 would have begun with its additional need for early stage tissues. In our original plan, Task 2 was to feed a large number of potentially overexpressed clones into Task 3 where this now reduced number of clones would have been screened with greater accuracy. The outcome of Task 3 would have been a very small number of clones which in turn would have been further analysed by sequencing, RT-PCR and Northern blot.

The accrual of bone marrow samples was similarly delayed due to procedural difficulties concerning patient consent. In order to not to deviate too much from the original plan or timeline, we decided to postpone the use of early stage tissues accrued through the Core until Task 3 would be implemented. Since this allowed more time for Task 2, we were able to use a more time-intensive protocol for the membrane hybridizations in order to yield more accurate hybridization results. We therefore included one early stage ovarian carcinoma from a previous collection through Swedish Hospital, and using the tissues that were available (in parenthesis the originally proposed numbers): 12 normal ovaries (9), 2 benign cystadenomas (4), 1 early stage (7), 12 late stage ovarian adenocarcinomas (7), 0 bone marrow samples (2), 1 liver (2), 3 peripheral blood lymphocytes (0) 1 peripheral blood lymphocyte cell culture (0).

We maximized the quality of hybridization results through use of more labor-intensive protocols, generated error assessment through triplicate hybridizations, and performed an in-depth analysis (including sequencing of selected clones) at this early stage rather than waiting for the glass array results in Task 3.

Work accomplished in Task 2:

The probe preparation, hybridization of the membranes and extraction of the hybridization intensities was performed as described earlier (Schummer 1999). The intensity for each cDNA hybridized with each tissue was stored in a database. This database contains the entries from 102,680 cDNAs and 47 hybridization events (32 tissues of which 5 had been hybridized 3 times and 2 twice, plus the hybridization with the vector probe and a "junk" probe recognizing housekeeping genes that are commonly overexpressed in tumors but have no relevance as markers). In addition, the database contains the patient information gathered during tissue accrual by the patient questionnaire, as well as the marker status for Her2/neu and p53 from the marker tests performed in the core laboratory. All in all the database contains more than 4.8 million records.

Once in a digital format, the data could be analyzed using the most recently developed algorithms for expression analysis. In collaboration with the computer science department at the University of Washington we initially employed statistical measurements to reduce the number of clones in this immense dataset from 100,000 to less than 3000 in order to be manageable by the clustering software written by Ben-Dor et al (Ben-Dor A 1999). Appendix A, Figure 2 shows how such an approach can reduce the number of clones to a manageable few. Also the selected clones exhibit a tendency to a higher expression in the tumor tissues as the brighter right half of the image in **B** implies. Table 2 lists the statistical algorithms that were employed for the reduction of the dataset.

		#accumulate to
ZScore > 10.09 in >1 of all tumors	1192	1192
t Statistic > 4.00	300	1476
avg(Tumor) > 2.5* avg(Normal)	277	1661
avg(Tumor) > 2.5* avg(Normal,PBL,liver)	624	2181
avg(zScore) > 1.4	1439	2949
Minus junk=1	298	2651

Table 2
Statistical analysis that led to the 2651 selected clones. Each statistical method selected a certain number of clones that added up to 2949. The "junk" probe was a probe consisting of 40 housekeeping genes (ribosomal proteins, mitochondrial genes, elongation factors) that were previously found to have elevated expression in carcinomas presumably due to the elevated metabolism. It reduced the number of clones by 298 to 1651.

The selected clones were clustered using Amir Ben-Dor's software and despite the necessary adjustments to our protocol the results were surprisingly satisfying:
a) Based on the expression patterns we could separate a large number of tumors from the normal tissues. Grouping of tumors does not correlate with stage or grade. Benign tumors may group together, however their number was too low for a definite answer (Appendix A, Figure 4).
b) Based on these clustering results we selected 2651 clones, which were sequenced and homology searched.
c) The expression patterns would group certain clones together that turned out to be either mostly copies of the same gene or genes with similar behavior. One such group contains the "gold standard" HE4 that was found earlier to be a potential marker gene for ovarian cancer (Schummer et al., 1999), together with other genes, among them SLPI, a secreted protease inhibitor just like HE4 (Appendix A, Figure 3). The overexpression of SLPI in tumors could be confirmed by other methods (RT-PCR). Other clones from this group show no match to any known gene and may be potential novel marker genes (Appendix A, Figure 5).
Of the 2500 clones, 883 represent unique genes (467 known genes, 51 with no match to known genes, and 365 ESTs) that are currently arrayed onto glass slides in preparation for Task 3 (further characterization of genes).

Project 2
Antibody Immunity to Cancer Related Proteins as a Serologic Marker for Ovarian Cancer
Nicole Urban, ScD, Brad Nelson, Ph.D., Mary L. Disis, MD

The goal of this project is to assemble a panel of known and novel ovarian tumor antigens that may form the basis of a cost-effective, serologic screening test for early stage ovarian tumors. Towards this goal, serum antibody responses to the known tumor antigens p53, HER2 and Myc are being evaluated by ELISA in patients with ovarian cancer versus normal controls. In addition, novel ovarian tumor antigens are being identified by an immunoscreening procedure known as SEREX. Novel antigens identified by SEREX will be further evaluated using purified proteins and larger numbers of normal, benign and cancer serum in an ELISA format. Statistical analyses will be employed to determine the sensitivity and specificity of the known and SEREX-defined tumor antigens for the detection of early-stage ovarian cancer.

Four major tasks were scheduled for the initial 24 months of this project as outlined in the Statement of Work. The progress on each task is presented below.

Task 1: Perform ELISA screens for p53, HER2, Myc. (Months 1-24)

A. To develop reproducible assays for detecting HER2 antibodies.

One of the keys to utilizing a laboratory-based assay in the analysis of large numbers of clinical samples is to establish the reproducibility and sensitivity of the assay. This has been accomplished using a capture ELISA to detect antibodies to the HER2 protein. A quality assurance program has been established for the generation of the cell lysates, use of coating antibodies and age or reagents used in the assay. These changes in procedure have greatly improved the reproducibility of the analysis. For initial studies we used sera collected form 157 volunteer blood donors derived from the Puget Sound Blood Center and sera from 50 patients with ovarian cancer and 50 patients with breast cancer. Sensitivity of the assay was assessed by determining the intra-assay coefficient of variation (CV) using positive control patients studied in 6 replicates on 10 plates. The intra-assay CV was 7% at the 1:25 dilution and 9% at the 1:100 dilution (CLIA standards recommend less than 10%). Precision of the assay was determined by assessing the inter-assay CV which entails analyzing the positive control sample for variation at multiple times over multiple assays. Over 63 plates, the inter-assay CV was 20% (CLIA standards recommend less than 10%). Finally, 50 random positive and negative samples as tested by ELISA were chosen for Western Blot validation to determine sensitivity and specificity. Defining the immunoblot as the "gold standard" the calculated specificity was 90% and sensitivity was 81%. Thus, the capture ELISA for the detection of HER2 specific antibodies is statistically adequate to begin wide-scale application of analysis of banked experimental samples.

Comparison of the capture ELISA to detect protein to two indirect ELISA methods each evaluating HER2 peptides as well as recombinant proteins was proposed. Analysis of 70 patients with either HER2 overexpressing breast or ovarian cancer as a test population, along with 100 volunteer donors, revealed the sensitivity of the peptide based assay compared to the capture ELISA using protein was less than 10%. No dominant B cell epitopes have been identified for HER2 and it is likely the response is polyclonal. No further studies of HER2 peptide based assays will be pursued. On the other hand, analysis of the same HER2 effected cancer population and volunteer donor pool indicates a 95% concordance of indirect ELISA using recombinant proteins with the capture ELISA utilizing human cell lines. Western blot validation to determine sensitivity and

specificity is currently being performed. Although the indirect ELISA using recombinant proteins is technically less difficult, the expense of generating and purifying these proteins is high. The proteins must be made from eukaryotic vectors as patients have baseline antibody responses to E. coli. Unless the sensitivity of the analysis was improved dramatically with the use of recombinant proteins, the standard for the rest of the studies will be the HER2 capture ELISA originally described in the proposal.

B. To develop reproducible assays for detecting p53 antibodies.

A capture assay defining p53 antibody response in a quantitative fashion has been established. ELISA plates (Dynex Technologies, Chantilly, VA) are coated with 50 ul of monoclonal antibody TIB 116 (20 ug/ml) in carbonate (1M Na2HC03/Na2C03) buffer. A row of IgG standards is also set up in carbonate buffer starting at a concentration of 2.0 ug/ml. The plates are incubated overnight then blocked with PBS/0.1% BSA/0.05% Tween-20 at 100 ul/well. BT-20 lysate at 50 ul/well is added to alternating rows and the plates are incubated overnight at 4°C. The plates are then washed with PBS-Tween and sera are added in dilutions from 1:25-1:200. After incubating for one hour at RT, the plates are again washed with PBS-Tween. Sheep anti-human Ig-HRP is added 1:5000 to all wells and plates are incubated at RT for one hour then washed with PBS-Tween. TMB-peroxidase is added as an enzyme substrate (75 ul) and the reaction is stopped with 75 ul/well 1N HCl when the OD of the IgG standard at 0.5 ug/ml reaches 0.3 at 650 nm. The plates are then read at 450 nm. This ELISA is completing sensitivity, specificity and precision studies as described for the HER2 assay. The population to be analyzed includes sera from 100 patients with ovarian cancer, stage III and stage IV as well as 160 volunteer donors.

In addition, two peptides defined as the dominant B-cell epitopes, identified from the literature, have been constructed for use in designing a p53 peptide ELISA. It is thought that the immunodominant parts of the p53 molecule are the amino and carboxy termini, as these are the hydrophilic segments located at the protein surface. A study published in 1994 analyzed p53 antibodies in the sera of lung cancer patients defined immunodominant regions in the p53 protein. 77 peptides consisting of overlapping 15-mers spanning the entire human p53 protein using an immunoblotting technique. Two immunodominant regions were localized in the amino terminus (peptide 3-5, EPPLSQETFSDLWKLLPENNVLSPL and peptide 9-10, DDLMLSPDDIEQWFTEDPGP). One epitope was generally located in the carboxy terminus and was more heterogeneous and sensitive to denaturation (peptides 70-77). For 5 patients, peptide scanning was also obtained after treatment and showed that the epitopes recognized by these sera had not changed, suggesting that p53 presentation is similar during the disease course. They also found that these amino and carboxy regions are totally devoid of any mutations in human cancers, indicating that the antibodies are directed towards p53 domains that are not altered by mutations. The conditions of analysis have been established for the p53 peptide assay and screening of the experimental populations described above has begun.

C. To develop reproducible assays for detecting c-myc antibodies.

The design of peptide and protein ELISA for c-myc is still under development. Following an extensive literature search, two different human c-myc B-cell epitopes have been identified from the literature (p305-318, HQHNYAAPPSTRK and p421-439, RKRREQLKEQLRNSCA). These peptides have now been constructed. A quantitative capture assay for detection of c-myc protein is still under development. In preliminary methods, plates are coated with CRL-1727 at 20 ug/ml in carbonate buffer, then incubated overnight. The plates are then blocked with PBS/1% BSA at 100 ul/well for 3-4 hours. The plates are then washed with PBS-Tween and HL-60 lysate in PBS/1% BSA is added to alternating rows at 50 ul/well. The plates are incubated overnight then washed in PBS-Tween. Serum dilutions are added at 50 ul/well and incubated I hour at RT. Anti-rabbit HRP at 1:5000 in PBS/1% BSA was added (50 ul) to each well and the plates incubated at RT for 45 minutes. TMB-

peroxidase as an enzyme substrate is added and the reaction stopped for a delta OD of 0.3 at 650 nm with 75 ul/well 1N HCl. The plates are then read at 450 nm. Sensitivity and specificity have not yet been validated using the peptide assay.

Initial results from the analysis reveal a high background of this assay, i.e. non-specific binding of sera to the coating antibody as well as inconsistent binding of the protein to the antibody in an ELISA format. Validation of interactions of the protein with the antibody have been performed by immunoblot. In addition, the coating antibody will immunoprecipitate the protein from a cell lysate. Troubleshooting methods to decrease non-specific while preserving specific antibody interactions are currently underway and include a change in incubation time and temperature, increasing the concentration of detergent in the buffer and experimenting with different blocking agents such as non-fat dry milk.

Task 2: Determine SEREX baseline (Months 1-6)

A. Conduct ten serial absorptions on sera from three normal individuals and three ovarian cancer patients with known reactivities to one or more of the p53, HER2/neu and Myc antigens.

The goal of this task was to optimize the signal-to-noise ratio of the SEREX protocol using a known ovarian tumor antigen. A control lambda phage clone expressing HER2/neu was constructed and mixed with a commercial lambda phage library. This "test" library was used to simulate a SEREX screen for ovarian tumor antigens. Human serum was spiked with a monoclonal antibody to HER2/neu and 0, 2, 4, 6 and 8 serial absorptions were performed. This experiment demonstrated that 2 serial absorptions yielded an optimal signal-to-noise ratio for detecting HER2/neu expressing lambda phage from a human cDNA library using human serum. While our original plan called for repeating this procedure with multiple serum samples, we believed a similar level of validation could be obtained by screening an actual tumor library with multiple patient sera. This deviation would allow us to continuously monitor signal-to-noise while potentially identifying some novel tumor antigens in the process. Since a suitable ovarian cDNA library was not commercially available, and we did not want to use our valuable (matched and characterized) ovarian cancer specimens for this work, we proceeded to screen a commercial breast tumor cell line cDNA library (ZR-75.1, Clontech) with serum from three women with breast cancer. Over 200 immunoreactive clones were identified during the primary screen. Secondary screening revealed that 5 of these showed a breast cancer-specific pattern of immunoreactivity and hence are novel candidate breast tumor antigens (Table 1). In addition to identifying candidate antigens relevant to breast cancer, these efforts also confirmed our original conclusion that two pre-absorption steps were optimal for pre-clearing serum of unwanted antibodies.

Table 1. Summary of serum antibody responses to five candidate breast cancer antigens (BCA#1-#5) among early- and late-stage cancer patients and normal controls. Individuals were scored as positive or negative for an antibody response to each antigen, as assessed by the phage array method described in the text. Shown are the number of positive responses in each group of individuals. Also listed are our internal clone numbers for each antigen, as well as the Genbank identifiers for two of the antigens.

Antigen Names			Immunoreactivity			
			Normals	Breast Cancers		
Name	Clone No.	Genbank Identifier	(n=17)	Total (n=26)	Early Stage (n=10)	Late Stage (n=16)
BCA#1	128.1	PENDING	0	4	2	2
BCA#2	31.1	gbT20211	0	4	1	3
BCA#3	62.3	PENDING	0	2	1	1
BCA#4	28.1	No match	1	5	2	3
BCA#5	26.2	gbT20172	0	1	1	0

B. Construct a cDNA expression library from pooled ovarian tumor samples.

After we had demonstrated that the procedure worked, ten stage III/IV serous ovarian tumors were used to construct a cDNA library using the lambda phage vector lambda TriplEx. Library construction proceeded as planned.

C. Assess the quality of the library.

Titration experiments demonstrated that the cDNA library contains $>1 \times 10^6$ primary clones. PCR analysis demonstrated > 95% recombinant phage and an average insert size of 1.7 kb. Partial sequencing of six randomly picked clones showed no evidence of genomic or bacterial DNA contamination. Most important, a primary screen with serum from a patient with stage III ovarian cancer has identified four clones encoding p53 (a known human ovarian tumor antigen), as well as three clones encoding a novel candidate ovarian tumor antigen (described below in task 3).

Task 3: Use SEREX to screen serum from ovarian cancer patients. (Months 6-20)

A. Identify novel ovarian tumor antigens.

Using the ovarian cancer cDNA library discussed above, we have screened 2×10^5 clones with serum from two ovarian cancer patients. These efforts resulted in the isolation of 137 immunoreactive clones, which were further evaluated in the experiments below.

B. Prioritize the evaluation of novel ovarian tumor antigens.

Of the 137 primary immunoreactive clones, 116 have been purified through a secondary SEREX screening procedure. These 116 purified clones have been evaluated for immunoreactivity to serum from multiple cases and controls using an array procedure developed within our laboratory. Serum from ten women with stage III/IV serous ovarian cancer and 15 age-matched women with no known history of cancer were tested for IgG, IgM and IgA responses to all 116 clones. An additional 20 patient and 15 normal samples will be evaluated in the near future. To date, fifteen clones with a cancer specific pattern of immunoreactivity have been identified. These clones were subcloned into plasmid vectors, purified and partially sequenced. Upon comparison with the human EST database and the Unigene database (NCBI), two distinct cDNA sequences were identified and are shown in Table 2 below.

Table 2. Summary of serum antibody responses to two candidate ovarian cancer antigens (OvCA#1 and #2) among ovarian cancer patients and normal controls. Individuals were scored as positive or negative for an antibody response to each antigen, as assessed by secondary SEREX screening. Shown are the number of positive responses in each group of individuals. Also listed are our internal clone numbers for each antigen, the number of times each gene product was identified in the primary screen, and a description of each gene product.

Antigen			Immunoreactivity	
Clone #	No. independent isolates	Description of gene product	Normal Controls (n=15)	Ovarian Cancer Patients (n=10)
OvCA#1	4	p53	0	2
OvCA#2	3	hZF5	0	2

		(zinc finger-containing protein)		

Two key points should be made regarding these results. First, the repeated cloning of p53, a known ovarian tumor antigen previously shown to induce auto-antibody responses, validates the quality of our library and our ability to identify bona fide tumor antigens using SEREX. Second, the identification of hZF5, a zinc finger protein of unknown function, provides our first candidate ovarian tumor antigen to be advanced for further validation by ELISA. The response rate of 2/10 seen for both p53 and hZF5 is typical for humoral tumor antigens (Stockard E et. al., *J.Exp.Med.* 187:1349-1354, 1998; Scanlan MJ et. al., *Int.J.Cancer* 76:1-7, 1998).

Task 4: Perform ELISA screens for Promising Candidates (Months 18-24)

While this task is not scheduled to begin for another 6 months, we expect to begin evaluation of hZF5 within the next month, provided it continues to look promising after secondary screening using an additional 20 patient and 15 normal sera. The hZF5 cDNA will be subcloned into the bacterial expression vector pQE and the eukarytoic expression vector pcDNA4/HisMAX. Recombinant protein will be purified from both bacterial and mammalian cells and transferred to the Disis lab for analysis by ELISA.

Task 5: Pool data for analysis. (Months 6-24)

This is ongoing. The Project Director has been summarizing the results to the Coordination Core at quarterly meetings. One promising gene discovered in Project 2 (human ZF5) will soon be provided to Project 1 for expression analysis in primary tumor specimens and to the Disis lab for ELISA analysis.

Statistical, Clinical and Laboratory Coordinating Core

Nicole Urban, ScD, Garnet Anderson, Ph.D., Nancy Kiviat, MD, Leona Holmberg, MD, Jane Kuypers, Ph.D., Charles Drescher, MD, Mary Anne Rossing, Ph.D.

The purpose of this shared resource is to support the work of the project investigators by collecting, storing and providing tissue and blood for analysis, as well as statistical analysis of results.

Task 1. Define data collection instruments: Months 1-3

During the pilot study period, data collection instruments were created to ensure that information needed for scientific research as well as that required for human subjects participation was obtained. The data collection instruments used by the Coordinating Core can be classified into three basic categories: Enrollment, Specimen Collection and Clinical Data Requirements.

Women who consent to participate in this study are provided an enrollment packet. This packet includes a study brochure, a combined participant enrollment form/medical records release form, and an informed consent form to be completed at the pre-operative office visit. A self-administered 20-minute questionnaire with a self-addressed, stamped envelope is also included in the enrollment packet. All enrollment materials are pre-labeled with a unique packet identification number and returned immediately to the study office and entered into the database, and where each participant will be given a unique participant number (UPN). These forms are labeled as Appendix B in the Appendices included with this annual report.

A brief self-administered questionnaire to be completed by all study participants was developed by the study epidemiologist, Mary Anne Rossing, Ph.D. The data collected focus on known or suspected epidemiological risk factors for ovarian cancer, including: menstrual and reproductive history; use of exogenous hormones (oral contraceptives and hormone replacement therapy); family and personal history of ovarian, breast and other cancers; sociodemographic factors; history of selected gynecologic surgeries including hysterectomy and tubal ligation; and other relevant medical conditions. The data collected will be sufficient to categorize women according to their menopausal status, estimated total years of ovulation, and prior personal and family history of cancer. Data will also allow an examination of the possible relation of various medical, hormonal, and reproductive factors with levels of various cancer screening markers of interest.

The UPN is used to label all data collection forms, requisition forms, and specimen transport forms. A unique 6-digit number is also used to label all specimens, with a duplicate of the label attached to the specimen collection form, blood processing form and specimen tracking form. These forms are labeled as Appendix C in the Appendices included with this report. The unique label number for each specimen will be linked to each participant's UPN in the specimen inventory database.

Extensive characterization is conducted for all specimens collected for this study. Clinical data collection forms were created to classify the specimens stored in the study repository. The study automatically receives a pathology report and operative notes on patients who provide specimens to this study. Dr. Charles Drescher, a co-investigator on this study conducts the first level of review by assigning a clinical diagnosis to the patient based on the pathology report and operative notes. A second level review is conducted by Dr. Nancy Kiviat at the Core facility whereby all formalin-fixed, paraffin-embedded specimens which correspond directly with the fresh frozen tissues are examined for histology. In addition to histology classification, the tissue is examined utilizing Immunohistochemistry techniques which is described in detail later in this report. A chart review is also conducted where relevant clinical data is abstracted and entered into the database system. The Data

Coordinator reviews the chart to abstract additional data regarding probable diagnosis prior to surgery; type and date of diagnostic tests performed and test results. Examples of these forms are included as Appendix D in the Appendices.

Task 2. Develop data management and tracking systems: Months 1-9

As part of this study, we have developed a relational database management system programmed in Visual FoxPro and Visual Basic to support all the tracking functions and store all the key scientific data associated with this project. This information system consists of two key components: 1) a participant enrollment database, and 2) a specimen inventory and data tracking system. Enrollment data, including personal contact information, is stored in the participant enrollment database. At the time of enrollment entry, the database generates a unique participant number (UPN) that is used in all subsequent correspondence regarding the study participant, her blood and tissue specimens, and the data associated with these specimens. Data from the medical history questionnaire that the participant completes at the time of enrollment is also entered in this module.

The specimen tracking system is a multi-functional application that tracks all specimens collected for the QUEST study as well as for ORCHID. As described below, bloods collected from women participating in QUEST are required as normal controls in ORCHID work to be carried out in Year Two. Each individual specimen container or vial is labeled with a unique 6-digit number at the time of collection; these numbers are in turn associated with the UPN of the donating participant and the date of collection at the time of data entry. Immunohistochemistry data and histology characterizations are also stored in this component. The specimen tracking system also allows for the entry of clinical data relevant to the distribution of tissue and blood specimens, including surgical pathology diagnoses, pre-operative CA 125 results, and other relevant clinical conditions. This system also serves as an inventory database allowing us to track the location of specimens in the freezers and those that have been sent to ORCHID project investigators.

Both components of the data management system reside on password-protected servers managed by the IS staff of the Cancer Prevention Research Program at Fred Hutchinson Cancer Research Center. In addition to the logging onto the network, a staff member entering the ORCHID system must supply a unique password to run the application.

Task 3. Collect liver and bone marrow specimens: Months 1-4

The objective of collecting liver and bone marrow specimens was to provide a non-ovarian control tissue to Project One for the High Density Array Hybridization (HDAH). The Hutchinson Center Institutional Review Board did not approve the collection of liver biopsy tissue for this study, and accordingly the protocol was modified to collect bone marrow specimens only.

Thirty ccs each of bone marrow was obtained from four consenting donors at the time of marrow harvest for family donor bone marrow transplant in the Clinical Division of the Hutchinson Center. Individual identifiers and patient identifying information were required for the study. This bone marrow was provided to Project One, where RNA was extracted from each bone marrow sample to be used in the High Density Array Hybridization. The collected marrow is used as a reference or control tissue to allow elimination of cDNA's corresponding to

genes/proteins which are highly expressed in hematopoetic tissue and hence expected to be found in either serum or blood cells.

Task 4. Recruit surgery patients: Months 1-24

Goal of 190-249 annually.

All patient recruitment for this study occurs at the office of Pacific Gynecology Specialist (PGS), which is located on the campus of Swedish Medical Center (SMC) in Seattle. PGS consists of eight gynecological surgeons including three who specialize in gynecological oncology. Approximately 60% of the PGS oncology practice occurs on the campus of SHMC, while the remainder is divided among seven community hospitals in the Puget Sound area. The staff for this study are housed at the Marsha Rivkin Center for Ovarian Cancer Research, which provides close proximity to and interaction with the clinical community and patient recruitment being conducted for the study. Also located nearby are Harborview Hospital Medical Center, Providence Medical Center, and Virginia Mason Medical Center.

All potential participants are invited to participate by the attending physician or study nurse at the time of the pre-operative office visit. Interested patients are provided for their review a brochure describing the study, known by its acronym ORCHID – Ovarian Research Collaboration Helping to Improve Detection. Should the patient agree to participate in the ORCHID study, which involves the provision of biological specimens, the attending physician, nurse or a research study staff member reviews the consent form and other required enrollment documents with the patient. Patients are given an opportunity to ask questions and address any concerns they may have about participating. Patients are informed that the decision to participate will not affect their treatment in any way, and that in agreeing to participate, they reserve a right to terminate their participation at any time without prior notice.

Research study staff complete the portion of the enrollment forms reserved for internal use only. This portion of the patient enrollment form indicates the date of scheduled surgery, institution of enrollment and identification of the enrolling physician, other medical or research study personnel. The enrolling staff member ensures that the patient has fully completed the informed consent, and notes in the patient's chart that she has been approached and has agreed to participate in the research study. To ensure that this patient will not be approached again about the study, the enrolling staff notes the patient's participation or refusal in the patient's medical chart. The completed enrollment forms are returned to the Marsha Rivkin Center. The Data Coordinator enters data from the enrollment forms into the study database on a daily basis. At the time that data from enrollment forms is entered into the study database, each participant is assigned a unique participant number (UPN). All self-administered questionnaires, after completion by the participants, are returned to the Core facility for editing and data entry.

Brochure Development

In order to increase study interest, and potentially recruitment, we have implemented the use of a study brochure. Our protocol states that interested patients will be provided written information describing the research study to review. A brochure was developed to serve as a more visually appealing document, and which addresses the questions we have found patients to ask during the course of enrollment.

Serum Only collections

An additional group of women who are undergoing surgery at other hospitals where tissue collection protocols have not yet been developed are being recruited to provide serum during the pre-operative office visit.

Collection of serum from this group of women began this year and is anticipated to capture an additional group of 120 women per year. To date 18 women have been enrolled in this component of the study.

Follow up process for data

With adherence to stringent enrollment procedures, very little participant follow-up for this study was anticipated, however on occasion follow-up has been required if a patient has not fully completed enrollment forms or has not returned the study questionnaire. A protocol to address such circumstances was developed. In such situations, a written request, followed by one telephone call, is made by the Study Coordinator. A letter is mailed to the participant if their enrollment materials are incomplete or if their questionnaire has not been received by the Core within thirty days of their enrollment. After fourteen days, a follow up call is made to the participant if she has not responded to the request. An example of this letter and follow up telephone script is included with this report as Appendix E.

Expansion of enrollment to Pacific Medical Clinics and Providence Seattle Medical Center

We have expanded our study enrollment locations to include the PacMed Women's Medical Specialists Clinic located in the Nordstrom Medical Tower on First Hill, at 1229 Madison Street – Suite 1450. PacMed Women's Medical Specialists surgeons conduct ovarian related surgery at Swedish Medical Center campus each Wednesday. An orientation has been conducted at this clinic, and physicians and clinical staff have agreed to participate in the ORCHID study. In addition, this clinic serves as a site of enrollment for other research studies involving human subjects. As is procedure currently, the patient is identified and approached by their physician or nurse about participating in the study and will be provided a study brochure to review. Only those patients scheduled to undergo ovarian related surgery at Swedish Medical Center by PacMed Women's Medical Specialists surgeons are considered eligible for this study. If the patient agrees to participate, an ORCHID study staff member is contacted to review the consent form and enrollment paperwork with the patient. The patient's physician is also available to answer any questions related to this study.

Another manner in which we are working to increase study enrollment is to conduct surgical tissue collections from PGS patients undergoing ovarian related surgery at Providence Seattle Medical Center. PGS surgeons perform surgeries at multiple locations in the Seattle metropolitan area. Previously, a portion of the patient population at PGS is scheduled to undergo ovarian related surgery, but are not available to participate in the ORCHID study because their surgery takes place at location outside of the Swedish Medical Center Campus, and thus we are not able to collect tissue. Providence Medical Center Seattle is one hospital where PGS surgeons operate. By expanding tissue collection activities to include this hospital as a site of enrollment, this population of patients may also be able to participate in the ORCHID study. As is current protocol, these patients are identified at PGS using current ORCHID study patient identification protocols, and are asked to complete similar enrollment materials. Only those patients identified by PGS physicians or clinical staff, AND scheduled to undergo ovarian related surgery at Swedish Medical Center or Providence Medical Center Seattle by PGS surgeons are eligible for this protocol.

Task 5. Collect tissue and blood specimens from surgery patients: Months 1-24

As of October 22, 1999, 217 women have consented to this study and successful ovarian tissue collections have occurred on 146, of which 39 patients were diagnosed with ovarian cancer, 6 with tumors of Low Malignant Potential, 31 with benign disease, and 57 with no ovarian abnormalities. (Investigators are awaiting pathology reports on the remaining 13 collections and 17 collections were conducted with non-ovarian primaries). The

cancer cases include 9 patients with early stage disease but unfortunately to date we have not collected usable tissue from any women with early-stage serous tumors.

Blood specimens are collected from all ORCHID study patients (40 ccs), if the anesthesiologist feels that the patient is in sufficient position to provide blood. We have learned that sometimes the patient is compromised and is not able to provide blood during the surgery thus we have implemented a protocol where the study tissue collection specialist coordinates with the pre-operative holding area nursing staff and collects blood for the study prior to surgery. If the patient is not compromised or undergoing extensive blood loss, the anesthesiologist on staff collects the blood, which is then taken to the respective hospital's clinical laboratory for processing into serum, plasma, and white blood cell pellets. In addition to these collections, blood is drawn from consenting women who have been diagnosed with operable ovarian cancer, but who will have surgery at a non-participating hospital. This blood collection occurs during the participant's pre-operative office visit in the offices of Pacific Gynecology Specialists. The blood is processed into sera, WBC pellets and plasma.

A dedicated tissue collection specialist is responsible for collection of specimens from ORCHID study patients. The staff member coordinates with Pacific Gynecology Specialists to maintain a surgery schedule of patients who have consented to participate in ORCHID research. At the time of each surgery, the Tissue Collection Specialist reports to the surgical suite with the signed consent forms and all necessary supplies. She requests coordinates with operating room personnel to collect biological tissue specimens within 30 minutes of removal from the patient. Under the direction of a clinical pathologist, tissue necessary for clinical evaluation is removed, after which the Tissue Collection Specialist requests tissue for the study. This request is filled at the pathologist's discretion and only after the diagnostic needs of the case are met.

Surgical specimens are placed in labeled sterile containers containing 0.9% sodium chloride and transported by the Tissue Collection Specialist to the processing area located in the frozen section room. Tissue used for the ORCHID study is selected from an area representative of the specimen and as free of necrosis as possible. In addition to primary ovarian cancer tissue, metastatic tissue with its site noted and uninvolved tissue from the contralateral ovary is also collected if available. Immediately after collection, each tissue specimen is labeled with a unique 6-digit numeric label. A duplicate of this label is affixed to a section of the participant's specimen collection form corresponding to the type and preparation of the tissue specimen. The Specimen Collection Form, included as Appendix C details the types of tissue, as well as the medium by which they are collected.

Up to one and five grams of each tissue type mentioned previously is usually collected. Each tissue specimen is divided into sections of approximately 1 cm^3. These sections are completely wrapped in aluminum foil and immersed in liquid nitrogen for a minimum of 3 minutes. The foil wrap containing tissue is then placed in a plastic tissue cassette labeled with a unique 6-digit numeric label for tracking. A duplicate of this label is placed on a specimen collection form like that described under the data collection instruments section. The label is affixed to the section of the form corresponding to the specimen type. Specimens are transferred to the Core Lab in liquid nitrogen. For each OCT specimen, a truncated mold is partially filled with OCT medium and pre-cooled by holding over (not in) liquid nitrogen until the OCT medium loses transparency. Specimens are placed into plastic biohazard bags labeled with a unique 6-digit number and stored in liquid nitrogen for transport to the Core repository. Formalin-fixed specimens are also collected and stored in the Core laboratory, where stained histology slides and sections for immunohistochemical analyses are made from them.

All frozen tissue specimens are stored in a liquid nitrogen tank at the Core laboratory. This tank is checked and filled with additional liquid nitrogen each week and is equipped with an alarm that sounds if the fluid level drops below a predefined volume. OCT specimens will be stored in boxes in a -70° C freezer. Formalin-fixed specimens are stored at room temperature in the Core laboratory. At the time of deposition in the repository, the well location, box identity, and freezer location of each frozen specimen are noted in an inventory notebook. Following each deposition, information from this notebook is used to update the specimen record in the specimen tracking database.

Specimen Allocation Procedures

After characterization in the Laboratory Core, specimens are made available to Project Investigators. After Project needs have been met, specimens may be made available to non-Project Investigators. In such circumstances, the non-Project Investigators will be required to complete a review process for use of said specimens. All specimens transferred to non-Project Investigators must receive approval and/or certification from Study Investigators, and the FHCRC Institutional Review Office (IRO). Specimens provided to commercial entities, or Investigators in collaboration with a commercial entities must also receive approval from the FHCRC Human Specimens Committee.

Non-Project Investigators and/or commercial entities will be asked to submit a proposal to this study's Investigators, stating the following: 1) the hypothesis to be tested 2) how the specimens will be used, 3) the amounts and types of specimens requested and 4) preliminary data. In addition, a biostatistical consult will be conducted to ensure that sample sizes are sufficient and that the study is sound in design.

If approved by study Investigators, non-Project Investigator(s) and/or commercial entities will be required to submit an Institutional Review Board application to the FHCRC IRO for research protocol review and approval.

If approved by the FHCRC IRO, and if not a commercial entity, or an investigator(s) involved in collaboration with a commercial entity, the specimen request will be considered approved. If approved by the FHCRC IRO, and if a commercial entity, or an investigator(s) in a collaboration with a commercial entity, non -Project Investigators will be required to submit application to the FHCRC Human Specimens Committee for research protocol review and approval.

Specimen Transfer

For all specimen transfers, a report identifying those specimens to be distributed is generated in the specimen inventory database. The investigator's name, laboratory location, and intended use is recorded in the database with the specimens (individually identified) to be sent to the research project. The Tissue Collection Specialist receives a copy of this delivery report, removes the specimens from the repository, and packages the specimens securely for transport to the investigator's laboratory. To ensure the integrity of the specimens, the freezer boxes will not be removed from the freezer for processing until all transport supplies are available for performing the transport procedure. The specimens are packaged in a styrofoam box according to study protocol.

Upon receipt of the delivery, the investigator and Tissue Collection Specialist will review the contents of the delivery and check them against the printed report. Both will sign a transmittal form confirming that the specimens listed were received in full and in satisfactory condition. The completion of this form and confirmation of delivery will be stored in the specimen database and linked to the records of the specimens

comprising the delivery. An example of this form is included with the specimen collection and tracking forms in Appendix C.

Task 6. Collect blood specimens from QUEST participants: Months 1-24

A portion of the blood specimens to be used as positive controls in the ORCHID study are to be obtained from women enrolled in the QUEST study. A total of 480 women are currently being randomized to this study, of whom 240 will be assigned to the intensive screening intervention arm. Women in this arm are being consented for additional blood to be drawn for research purposes, including this study. To date, the QUEST study has drawn bloods on approximately 75 women. The QUEST bloods are inventoried and labeled in the same manner as bloods collected for the ORCHID study, and are stored in the same repository as the ORCHID bloods.

Task 7. Collect epidemiologic and clinical data: Months 1-24

Epidemiologic and clinical data collection is ongoing in the ORCHID study. Each week, study staff are able to generate a report showing successful collections without other required data (questionnaires, core histologic review, clinical data records etc). For self-administered forms, the project coordinator follows procedures outlined in the Follow Up Protocol described previously. For clinical information, a clinical data follow up reported is created by the study database. The Data Coordinator visits PGS to review the patients' charts to obtain detailed information on final diagnosis. A copy of the final pathology report is automatically obtained from Dynacare Laboratory of Pathology and included in the participant's study file. Data from the Core Laboratory is entered at the laboratory and submitted bi-weekly in electronic format for inclusion in the database.

As stated previously, data collected from the questionnaire will be sufficient to categorize women according to their menopausal status, estimated total years of ovulation, and prior personal and family history of cancer. Data will also allow an examination of the possible relation of various medical, hormonal, and reproductive factors with levels of various cancer screening markers of interest. As of October 1999, 184 (84% of total enrollment) questionnaires have been completed and returned to the study office.

As described previously, clinical follow-up data is collected by the Data Coordinator via review of the participant's medical records using standardized forms developed by the Core investigators, and entered into the clinical database by the Data Coordinator. This data includes selected information on disease characteristics including diagnostic test results, histology, stage, grade, tumor distribution, extent of residual disease and any other standardized data as determined by Core investigators. In addition, the Data Coordinator will conduct quarterly follow-up regarding chemotherapy administered, response to treatment, disease status and survival.

Task 8. Characterize histology of tissue specimens: Months 1-24

Dr. Kiviat is responsible for conducting a pathology review of each tissue specimen collected during surgery. The results of this characterization are coded and associated with each individual tissue specimen in the specimen inventory database.

During months 1 through 12, histological examination was carried out on 127 cases and classified according to the World Health Organization (WHO) classification of ovarian tumors. Cases identified as tumors (benign or malignant) or other epithelial lesions were further characterized by immunohistochemistry.

Task 9. Perform tissue assays for p53, Her-2/neu, and c-myc: Months 3-24

The core laboratory supporting this study conducts assays for the oncoproteins Myc, HER2/*neu*, and p53 in each malignant tissue and a fraction of all normal tissues. In addition, p53 proteins are isolated and analyzed for mutation. The results are generated by the Core laboratory and are compiled and reported monthly to the study investigators. In addition to keeping investigators abreast of ongoing laboratory activity, this report serves as a quality control measure that would reveal problems with screening assays or methods of tissue collection and processing.

Seventy-two cases were characterized by immunohistochemistry. A panel of three antibodies was run on each case. Tissue reactivity was assessed using a monoclonal antibody directed against cytokeratin 8 (Becton Dickenson). To identify p53 overexpression, a monoclonal antibody which reacts with both the wild and mutant form of p53 was used (DAKO Corporation). Tumors that overexpressed the cerbB-2 oncogene product were identified using a polyclonal antibody (DAKO Corporation). It was originally proposed that a group of 15 cases consisting of high and low grade tumors and normal ovarian tissue were to be characterized by the polyclonal antibody directed against the mutant form of EGFR (EGFRvIII). Due to the low cost of running an additional slide, it was decided that all prospective cases will be characterized with EGFRvIII as well as any retrospective cases in which unstained slides are available. These cases will be run beginning in month 12.

Thirty cases consisting of normal, benign, and malignant tissues were characterized using two antibodies directed against c-myc oncogene product. From the results it was determined that the antibodies were non-specific and staining was very fixation dependent. It was decided to put a hold on pursuing c-myc until a more specific and reliable antibody can be found.

The results of the histological and immunohistochemical characterization of the tumors and lesions are shown below:

Normal	# of Cases	p53 +	p53 -	cerbB-2 +	cerbB-2 -
Normal Ovarian Tissue[1]	41	-	-	-	-

Comment:
[1]Immunohistochemistry not run on these cases

Non-neoplastic Lesions	# of Cases	P53 +	P53 -	cerbB-2 +	cerbB-2 -
Benign cyst, not paraovarian[1]	3	0	3	0	2
Benign cyst, paraovarian	1	0	1	0	1
Benign dermoid cyst[2]	1	0	1	0	0
Endometriotic cyst[3]	4	0	4	0	3
Functional cyst[4]	10	-	-	-	-
Corpus luteum[5]	1	-	-	-	-

Comment:
[1,2,3]1 case was indeterminate for cerbB-2

[4]Immunohistochemistry not run on these cases
[5]Immunohistochemistry not run on these cases

Non-epithelial Neoplams	# of Cases	p53 +	p53 -	cerbB-2 +	cerbB-2 -
Ovarian fibroma[1]	3	-	-	-	-
Thecoma[2]	2	-	-	-	-

Comments:
[1]Immunohistochemistry not run on these cases
[2]Immunohistochemistry not run on these cases

Brenner's Tumor, Benign	# of Cases	p53 +	P53 -	cerbB-2 +	cerbB-2 -
Benign Brenner's tumor, typical	1	0	1	0	1

Serous Tumors, Benign	# of Cases	p53 +	P53 -	cerbB-2 +	cerbB-2 -
Serous cystadenoma	3	0	3	0	3
Serous cystadenofibroma	3	0	3	0	3
Serous adenofibroma	2	0	2	0	2

Serous Tumors, Low Malignant Potential	# of Cases	p53 +	P53 -	cerbB-2 +	cerbB-2 -
Serous Carcinoma of LMP[1]	4	0	3	0	2

Comment:

[1]1 case was equivocal for p53 and 2 cases were indeterminate for cerbB-2

Serous Tumors, Malignant	# of Cases	p53 +	P53 -	cerbB-2 +	cerbB-2 -
Serous carcinoma[1]	20	14	5	2	5

Comment:
[1]1 case was equivocal for p53 and 13 were indeterminate for cerbB-2

Mucinous Tumors, Benign	# of Cases	p53 +	P53 -	cerbB-2 +	cerbB-2 -
Mucinous cystadenofibroma[1]	1	0	1	0	0
Mucinous cystadenoma[2]	3	0	3	0	0

Comment:
[1] 1 case was indeterminate for cerbB-2
[2] 3 cases were indeterminate for cerbB-2

Mucinous Tumors, Low Malignant Potential	# of Cases	p53 +	P53 -	cerbB-2 +	cerbB-2 -
Mucinous carcinoma of LMP[1]	2	0	2	0	0

Comment:
[1] 2cases were indeterminate for cerbB-2

Mucinous Tumors, Malignant	# of Cases	p53 +	P53 -	cerbB-2 +	cerbB-2 -
Mucinous carcinoma[1]	3	2	0	1	0

Comment:
[1] 1 case was equivocal for p53 and 2 were indeterminate for cerbB-2.

Endometrioid Tumors, Malignant	# of Cases	p53 +	P53 -	cerbB-2 +	cerbB-2 -
Endometrioid Adenocarcinoma[1]	3	1	0	1	1

Comment:
[1] 2 cases were equivocal for p53 and 1 was indeterminate for cerbB-2.

Clear Cell Tumors, Malignant	# of Cases	p53 +	P53 -	cerbB-2 +	cerbB-2 -
Clear cell carcinoma[1]	3	0	2	1	0

Comment:
[1] 1 case was equivocal for p53 and 2 cases were indeterminate for cerbB-2

Other, Malignant	# of Cases	p53 +	P53 -	cerbB-2 +	cerbB-2 -
Adenocarcinoma, NOS[1]	7	6	1	0	0
Unclassified epithelial tumor[2]	5	2	3	1	0
Endometrial adenocarcinoma	1	0	1	0	1
Metastatic colonic carcinoma	1	1	0	0	0

Comment:
[1]7 cases were indeterminate for cerbB-2
[2]4 cases were indeterminate for cerbB-2

Of the seventy-two cases characterized by immunohistochemistry, six tumors were determined to be equivocal for p53. Thirty-eight cases were indeterminate for cerbB-2. These specimens either did not contain normal epithelial tissue or a normal epithelial control was not available. To remedy this situation, the immunohistochemical assay will be repeated on the "clinical" normal and tumor specimens to be obtained from Dynacare Laboratories. The equivocal p53 cases will also be repeated on the Dynacare material in the event that the results were fixation related.

In the group of cases where primary and metastatic tissue were collected and characterized by immunohistochemistry, there were no differences in the p53 and cerbB-2 results between the primary and metastatic tumors (data not shown). With the exception of those cases that were identified as indeterminate for cerbB-2, none of the benign tumors or lesions displayed overexpression of either p53 or cerbB-2.

Task 10. Perform serum assays for CA-125: Months 3-24

Serum assays for CA-125 have not yet been performed. In the interest of preserving these very well characterized sera, the Investigators in this study decided not to conduct CA-125 serum assay measurements until the sera could be thawed and used by the projects at the same time.

Task 11. Supply Project 1 with 6 specimens to construct cDNA libraries: Months 1-2.

A total of 15 specimens were provided to Project 1 for the construction of cDNA libraries. This task has been completed and is described in greater detail in the Project 1 section of this report.

Task 12. Supply Project 1 with 30 specimens to hybridize with first generation membranes: Month 5.

A total of 31 specimens were provided to Project 1 to hybridize with the first-generation membranes. This task has been completed and is described in greater detail in the Project 1 section of this report.

Task 13. Develop statistical algorithms for selecting over-expressed genes for subsequent efforts: Months 4-21.

This task has not yet been started. We are currently working on upgrading our computing facilities at FHCRC to support analysis of large data sets, and in the mean time the analyses for pertaining to Project 1 have been

completed at the University of Washington as described in Project 1. We don't really need to do these analyses until we finish the hybridizations.

Task 14. Supply Project 1 with 105 specimens to hybridize with second-generation membranes: Month 11.

This task is expected to begin in month 13. The sequencing of the genes identified during the first generation hybridization took longer than expected.

Task 15. Supply Project 2 with 10 ovarian tumor samples to construct cDNA expression library (Aim 2): Months 1-6:

This task is completed. Collection of early stage serous tissue specimens has been lower than expected. Project 2 was provided with specimens from two ORCHID study participants and the remaining specimens were obtained from the Gynecologic Oncology Group.

Task 16. Supply Project 2 with 600 blinded samples for antibody response analyses (Aim 1):Months 3-24.

This task has not yet begun. Dr. Mary L. Disis is responsible for optimizing the assays involved in the antibody response analyses. The investigators involved in this study have elected to build up our specimen repository before providing samples to the Project for antibody analyses. These analyses will commence in month 20 of the study period.

Task 17. Supply Project 2 with tissue samples from ovarian cancer cases and controls for SEREX analyses (Aim 2): Months 6-12.

This task has not yet begun. Biological tissue samples from ovarian cancer cases and controls are to be allocated in month 13. This is described in detail in the Project 2 component of this report.

Task 18. Conduct statistical analysis of antibody response to Her-2/neu, p53 and c-myc (Project 2, Aim 1): Months 12-24

This task is to begin in Year 2.

Task 19. Conduct statistical analysis of Her-2/neu, p53 and c-myc expression in tissue: Months 12-24

This task is to begin in Year 2.

Task 20. Conduct statistical analyses of select clones with clinical, epidemiologic and other laboratory data: Months 21-24.

This task is to begin in Year 2.

KEY RESEARCH ACCOMPLISHMENTS:

Project 1

- Creation of a database containing all hybridization results.
- Identification of several genes with no previously ascribed role in ovarian cancer that exhibit a cancer-restricted expression behavior.

Project 2

Task 1

- Fully operational and reproducible assay for detection of HER2 antibodies for use in final analysis.
- Fully operational assay for the detection of p53 antibodies.
- Construction of peptides which are dominant B cell epitopes of p53 and c-myc.
- Development of conditions to detect peptide specific antibody responses by ELISA.

Task 2

- Successful optimization of the signal-to-noise ratio for the SEREX protocol.
- Construction of a serous ovarian tumor cDNA library.
- Development of an array based procedure that allows evaluation of up to 70 different clones with 30 different serum samples in three days.

Task 3

- Validation of the cDNA library and SEREX immuno-screening procedure by cloning the known ovarian tumor antigen p53.
- Cloning of one novel candidate ovarian tumor antigen (hZF5).

Core

- Creation of a participant database containing epidemiological and clinical data
- Creation of a specimen inventory tracking system containing characterization and analyses data

REPORTABLE OUTCOMES:

Project 1

A database containing more than 4.8 million entries from the hybridization of the cDNA arrays.

Schummer M, Ng WL, Bumgarner RE, Nelson PS, Schummer B, Hassell L, Rae Baldwin L, Karlan BY, and Hood L (1999) Comparative hybridization of an array of 21,500 ovarian cDNAs for the discovery of genes overexpressed in ovarian carcinomas, Gene **238**, 375-385

Zong Q, Schummer M, Hood L, Morris DR (1999) Messenger RNA Translation State: The Second Dimension of High-Throughput Expression Screening, Proc. Natl. Acad. Sci. USA **96**, 10632-10636

Ben-Dor A, Bruhn L, Friedman N, Nachman I, Schummer M and Yakhini Z, "Tissue Classification with Gene Expression Profiles". Submitted to "The Forth Annual International Conference on Computational Molecular Biology -- RECOMB'2000".

Proposal:
"Genes Associated with Chemoresponsiveness in Ovarian Cancer" (Hood, PI) part of ovarian SPORE grant.

Three presentations:

"Finding Genes that are Overexpressed in Chemoresistant Ovarian Carcinomas", M. Schummer and Charles Drescher, Marsha Rivkin Center for Ovarian Cancer, Seattle, January 1999

"Using High Density Array Hybridization to Discover Potential Marker Genes for Ovarian Cancer", Corixa Corp., Seattle, February 1999

"Using High Density Array Hybridization to Discover Potential Marker Genes for Ovarian Cancer", ZymoGenetics Inc., Seattle, April 1999

Project 2

No reportable outcomes have been generated to date. However, all goals that were established for months 1-18 have been met. In addition, we have isolated one novel candidate tumor antigen that may be the subject of future manuscripts.

Core

Development of an ovarian specimen repository housing over 3000 individually identified specimens.
Development of a participant database and specimen inventory tracking system.
Funding of the 1999 ovarian cancer Specialized Program of Research Excellence by the NCI.

CONCLUSIONS:

The work of the ORCHID study is progressing according to schedule. We have obtained some encouraging results despite some difficulties in accrual of patients for specimen collection. The reviewers of the proposal anticipated these difficulties but noted that the objectives of the research could be met with smaller numbers of patients. Of particular concern to us is the scarcity of early-stage serous tissue to support the needs of Project 1. We have begun recruitment of women from two additional hospitals in the community, in order to increase our chances of identifying cases of early-stage ovarian carcinoma of serous histology.

Project 1 investigators changed their protocol somewhat to accommodate the slow accrual and the dearth of early-stage serous tumor specimens. Generally, however, they have proceeded as described in the proposal and their work has been very productive. They have identified two potentially interesting genes already, and their hybridization work is not yet completed, so we anticipate having several candidate genes for assay development next year. Project 2 investigators have overcome two major stumbling blocks that were anticipated for successful SEREX immunoscreening. First, they have optimized methods for removal of E.coli-specific antibodies present in serum samples, while maintaining antibodies to known tumor antigens. In addition, they have successfully constructed and performed a primary SEREX screen with an ovarian tumor cDNA library. Finally, they have cloned p53, a known ovarian tumor antigen, along with hZF5, a novel candidate ovarian tumor antigen.

We are excited about our identification of HE4, SLP1 and hZF5 as potential markers of ovarian cancer. We will continue to identify more candidate genes next year as we conduct second-stage library probes, at the same time that we proceed with evaluation of the two genes and single antigen that look promising. We are eager to develop assays for these markers, as we have obtained funding from the National Cancer Institute for an ovarian cancer SPORE that includes two projects that will evaluate a panel of markers including these if they turn out to have good performance characteristics. Our goal is to identify a panel of markers that can be used to identify women at high risk for ovarian cancer in the future, as well as women who already have developing early-stage ovarian cancer.

REFERENCES:

Ben-Dor A, B. L., Friedman N, Nachman I, Schummer M and Yakhini Z, (1999). "Tissue Classification with Gene Expression Profiles." Submitted to "The Forth Annual International Conference on Computational Molecular Biology -- RECOMB'2000".

Nelson, P. e. a. (1998). "An expressed-sequence-tag database of the human prostate: sequence analysis of 1168 cDNA clones." Genomics **47**(1): 12-25.

Schummer, M. (1999). "Comparative Hybridization of an array of 21 500 ovarian cDNAs for the discovery of genes ovexpressed in ovarian carcinomas." Gene **238**: 375-385.

Zhang, L., W. Zhou, et al. (1997). "Gene Expression Profiles in Normal and Cancer Cells." Science **276**: 1268-1272.

Appendix A

Project 1 FIGURES

Identification of Potential Markers for Population Based Screening for Ovarian Cancer: Characterization of Differential Gene Expression in Malignant Neoplasia by Use of High Density Array Hybridization (HDAH).

Nicole Urban, ScD, Leroy Hood, PhD, MD,
Michel Schummer, PhD, Nancy Kiviat, MD

Figures for Project 1

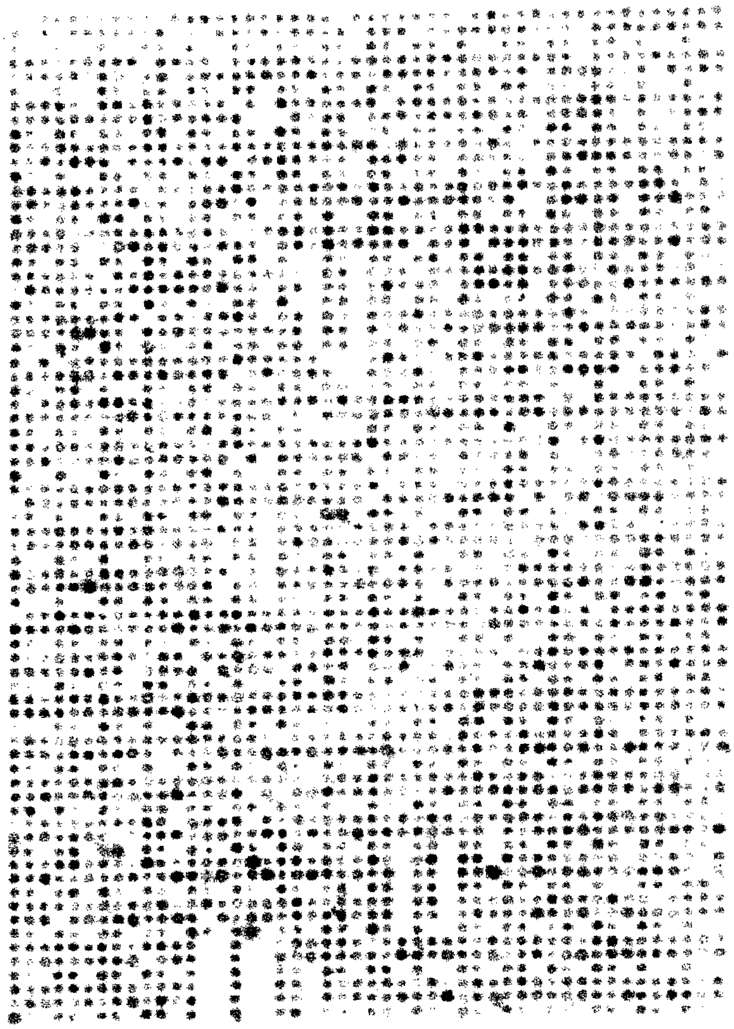


Figure 1

Close view on 1/6 of a membrane containing 3456 colonies that was hybridized with a probe regognising the vector portion of the cDNA. Where there is no signal, no colony grew. Overall, the number of colonies that did grow reaches 95%

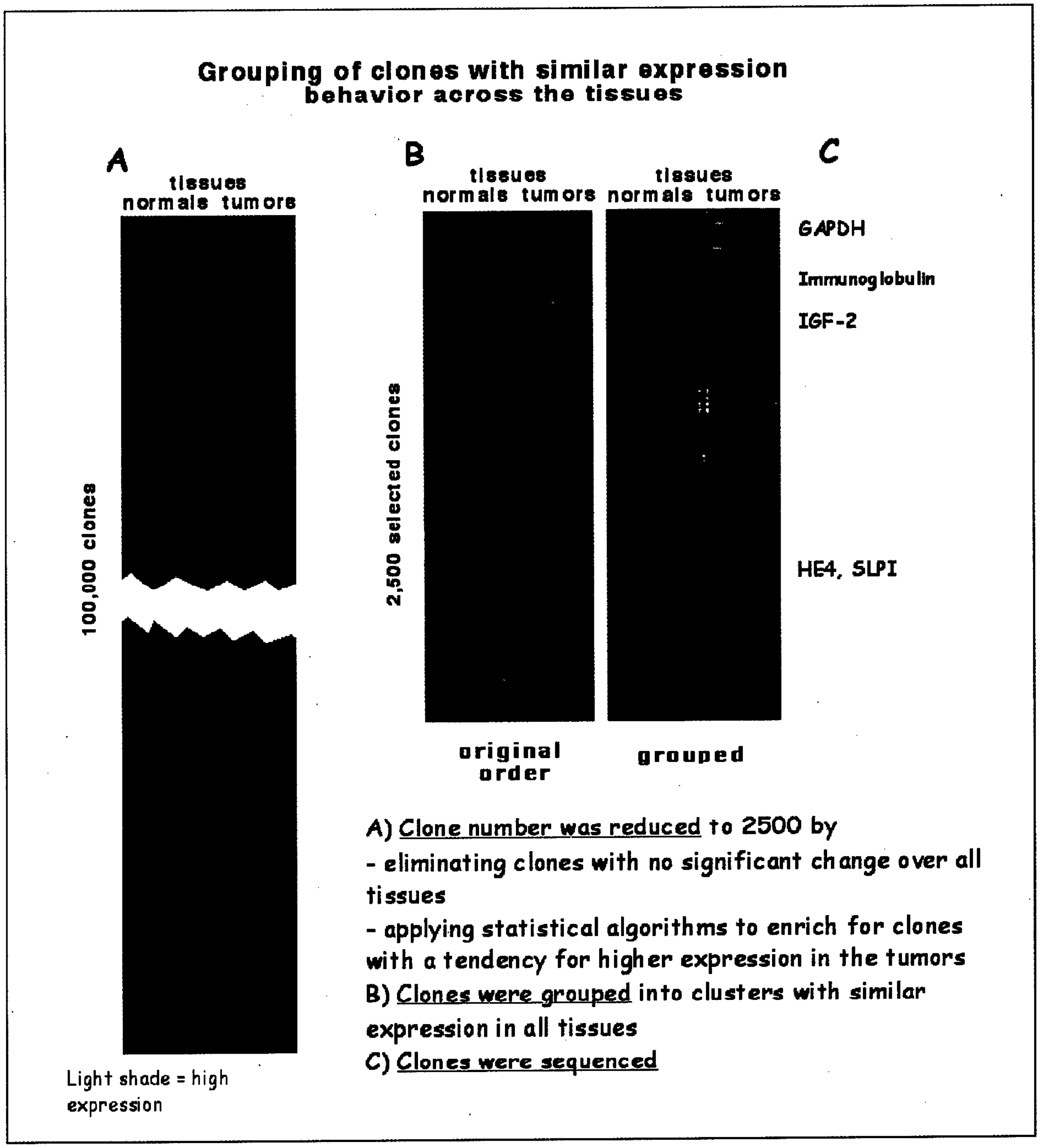
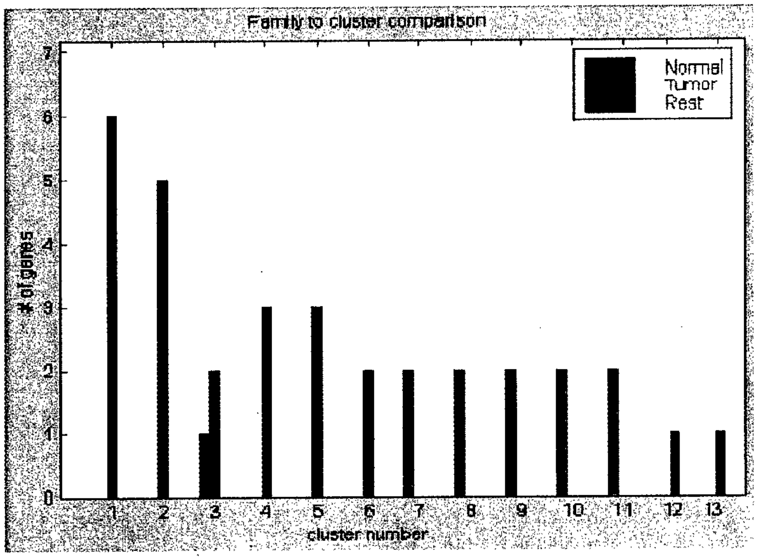


Figure 2
A Graphical representation of the hybridization patters of all 100,000 clones (y axis) in all tissues (x axis, normal ovaries on the left and tumors on the right). Bright color stands for a strong hybridization signal. **B LEFT** Graphical representation of the 2500 clones selected from the 100,000 by application of statistical algorithms that select for clones with a tendency to higher expression in the tumors. **B RIGHT** Graphical representation of the 2500 clones after grouping them into clusters with similar expression across the tissues using an algorithm that results in small cluster sizes. **C** Sequence identity of the majority (>80%) of the clones in some selected clusters.

Grouping of tissues based on expression of 2500 selected clones



1	PBL cult.	PBL culture
	t037	mucinous stage I
	t051	serous stage III
	t051a	serous stage III
	t040	serous stage IV
	t065	serous stage IV
2	t025	serous stage III
	t060	serous stage III
	t066	serous stage III
	t044a	serous stage IV
	t044b	serous stage IV
3	n050a	normal ovary
	t048	serous stage III
	t044	serous stage IV
4	t046	serous stage IV
	t046a	serous stage IV
	t046b	serous stage IV
5	t063	undifferentiated stage III
	t048a	serous stage III
	t048b	serous stage III
6	n039a	unknown stage I accomp.
	t043	serous stage III
7	PBL7+	PBL
	PBL8	PBL
8	n047a	normal ovary
	n047b	normal ovary
9	n050	normal ovary
	n050b	normal ovary
10	hliv2	liver
	PBL6	PBL
11	n056	normal ovary
	n064	normal ovary
12	t062	benign tumor
13	t058	benign tumor

Clustering was performed with the Software Bioclust (A. Ben-Dor et al.) with a cutoff value of 0.6670 based on the 2500 selected clones

Figure 4
Graphical display of the clustering result of the tissues used for hybridization. The bottom half lists the tissues and their stages. No correlation between stage and clustering is visible.

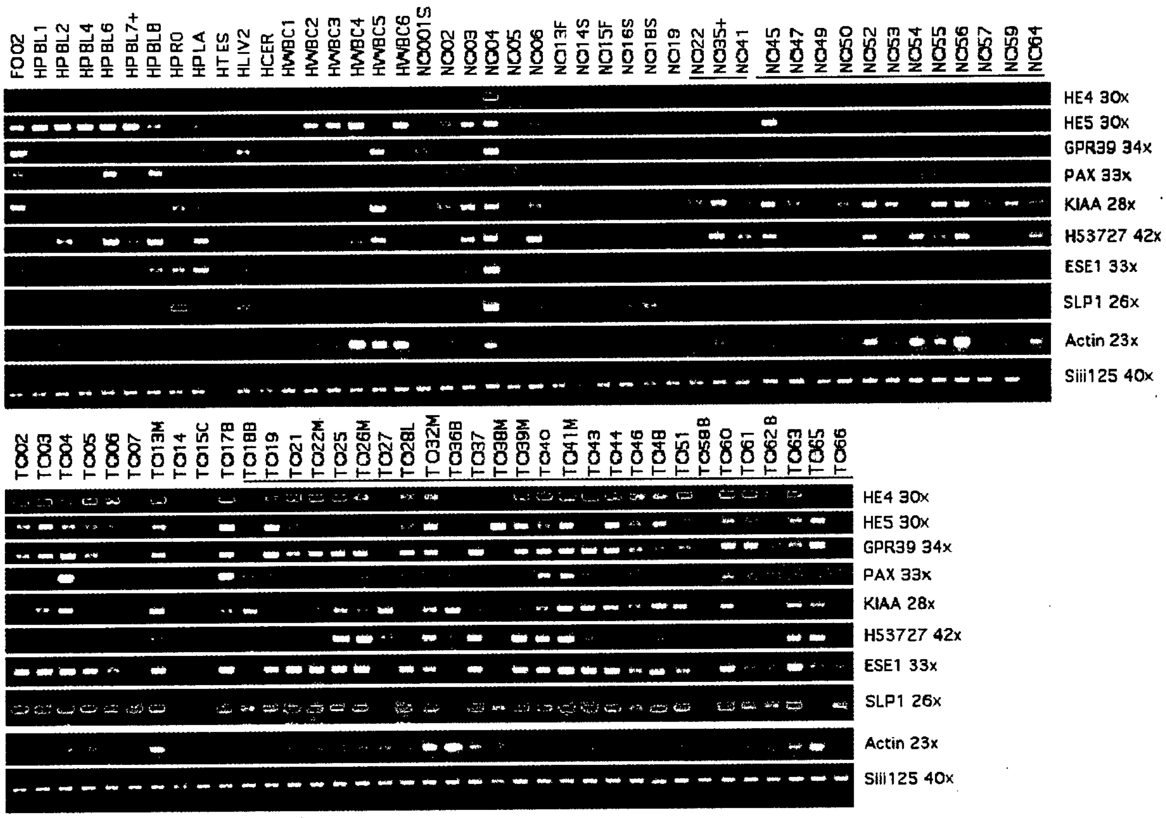


Figure 5
RT-PCR expression analysis of genes selected after cluster analysis. HE4, SLPI, GPR39, ESE1 and PAX exhibit a striking tumor-restricted expression behavior. Beta actin also shows differential expression. Unfortunately, theclones with no homology to known genes, KIAA and H53727, do express in normal ovarain tissues and are hence not suited as markers.

Appendix B

Core Enrollment Forms

Statistical, Clinical and Laboratory Coordinating Core

Nicole Urban, ScD, Garnet Anderson, PhD, Nancy Kiviat, MD,
Leona Holmberg, MD, Jane Kuypers, PhD,
Charles Drescher, MD, Mary Anne Rossing, PhD

"Use of Novel Technologies to Identify and Investigate Molecular Markers for Ovarian Cancer Screening and Prevention"

CONSENT TO PARTICIPATE IN RESEARCH STUDY:

ORCHID

Ovarian Research Collaboration Helping to Improve Detection

Conducted by Investigators at the Fred Hutchinson Cancer Research Center (FHCRC), University of Washington (UW) and Virginia Mason Research Center (VM). Funding by the Marsha Rivkin Center for Ovarian Cancer Research and the United States Army Medical Research and Materiel Command (USAMRMC).

Principal Investigator:
Nicole D. Urban, ScD
Fred Hutchinson Cancer Research Center
1100 Fairview Avenue North - MP-804
PO Box 19024
Seattle, WA 98109
(206) 667-4677

Investigators:
Garnet Anderson, PhD (FHCRC)
Nancy Kiviat, MD (UW)
Leroy Hood, MD, PhD (UW)
Brad Nelson, PhD (VM)
Charles Drescher, MD (FHCRC)
Mary Anne Rossing, PhD (FHCRC)
Jane Kuypers, PhD (UW)
Michel Schummer, PhD (UW)
Nora Disis, MD (UW)
Leona Holmberg, MD (FHCRC)

Location(s) of Study:

Fred Hutchinson Cancer Research Center (FHCRC)
1100 Fairview Avenue North
Seattle, WA 98109
(206) 667-5000

Pacific Gynecology Specialists
1229 Madison Street, #1050
Seattle, WA 98104
(206) 587-0585

Virginia Mason Research Center (VM)
1000 Seneca Street
Seattle, WA 98101

Marsha Rivkin Center for Ovarian Cancer Research
1221 Madison Street, Suite 1410
Seattle, WA 98104
(206) 386-2419

University of Washington (UW)
School of Medicine
Seattle, WA 98195
(206) 543-2100

PURPOSE

The goal of this research study is to establish a repository of gynecologic specimens that can be used to develop new screening tests to detect ovarian cancer before it spreads outside the ovary and becomes very difficult to cure. Investigators (research doctors) will study tumor tissue and blood from women with ovarian cancer, women with benign ovarian conditions, and women with no ovarian disease in order to identify genetic changes, or other changes that can be found in the blood, that are associated only with ovarian cancer. Results of this research will be used to develop methods for early diagnosis and prevention of ovarian cancer or other gynecologic cancers, and/or to provide better treatment for ovarian cancer. In addition, it will help scientists to better understand the biology of ovarian tumors.

WHAT YOU ARE ASKED TO DO

We are asking all eligible women to consider making their tissue and/or blood specimens available to investigators to carry out this research, which includes analysis of the cells at the molecular level (i.e., the smallest particles or components of the cell). This study is designed to look at differences between normal cells and the genetically altered cells that may give rise to cancer. These genetic factors may be inherited or they may be changes that result from environmental exposures. If you agree to participate in this study, a small amount of your white blood cells may be "grown" in the laboratory to develop a "cell line" that can be used for research.

If you agree to participate in this study, you can opt to participate in one of two ways:

A) ***If you are scheduled to undergo ovarian related surgery at Swedish Medical Center*** you will be asked to provide ovarian tissue and blood samples. If you participate, your surgery will be conducted exactly as if you were not going to participate. If you participate, you will NOT have a more extensive surgery. Obtaining additional tumor or normal tissue and blood will not effect the length of your procedure.

B) ***If you are scheduled to undergo ovarian related surgery outside of Swedish Medical Center Campus*** you will be asked to provide blood samples only at the time of your pre-operative office visit at Pacific Gynecology Specialists.

Acceptance or rejection of your participation in this study will in no way affect the treatment you receive for your condition, nor will it affect the outcome of your treatment. With the exception of the surgical procedure (Option A), or blood draw (Option B), the actual duration of your participation in this study will vary depending on how much time is needed to 1) provide information and answer questions about this research to your satisfaction, 2) fill out consent forms, and 3) complete the 20-minute questionnaire. The total time involved exclusively for this study should not exceed 30 minutes.

PROCEDURES

In order to obtain the best results possible, you will be asked to provide the following information and specimens for this research study:

Questionnaire. All women who are scheduled to undergo surgery involving at least one ovary are considered eligible for this study. If you agree to participate in this study, you will be given a questionnaire (with a postage paid return envelope), which should take approximately 20 minutes to complete. This may be completed in the office during your pre-surgical visit, or at home at your own convenience. The data collected in this questionnaire will focus on known or suspected risk factors for ovarian cancer, and will include questions on menstrual and reproductive history, birth control history, family and personal history of ovarian, breast and other cancers and sociodemographic factors.

Medical Records. All participants in this study will be asked to provide copies of their medical records to the study. Relevant medical record information will be obtained from your physician's office. You will be asked to complete a medical record release to authorize access of research personnel to this information. Relevant information, such as prior history of cancer and treatment, will be entered into the study database.

Blood draw.

Option A:
At the time of your surgery, your anesthesiologist will collect up to 40cc of blood (approximately 3-4 tablespoons) to be used in this research.

Option B:

At the time of your pre-operative office visit, a certified phlebotomist will collect up to 40cc of blood (approximately 3-4 tablespoons) to be used in this research.

Tissue Collection.

Option A only:

During surgery, your surgeon will first remove the tumor (if applicable) or benign ovarian tissue (if applicable). After this suspect tissue has been removed, the surgeon will allow the tissue collection specialist to collect small pieces of the suspect tissue and surrounding normal tissue that were removed during your operation. A certain amount of tumor tissue is needed to assist your surgeon in making a diagnosis. If there is not enough tumor specimen to both make a diagnosis and contribute to the research study, all of your tissue will be used to make a diagnosis. In that case, no tissue specimen will be provided to the study tissue collection technician, and you will not be included in this study. If there is enough tumor specimen to both make a diagnosis and contribute to the research study, the tissue specimen, along with the blood sample, will be sent to the Lab of Pathology for initial analysis, and then to the study specimen bank for further analysis and storage.

Research. The blood and tissue specimens will be stored at the designated study repository in Seattle, Washington for an indefinite period of time. The specimens will then be requested by laboratory investigators for molecular analysis. The results of these analyses will not be released to you or your personal care provider. These tests are experimental and are of unknown value in the diagnosis and treatment of ovarian cancer.

RISKS

Option A
Any tissue that is obtained for the purposes of this research study is collected only after it has been removed for the purposes of the surgical procedure that you are receiving. Obtaining blood prior to the beginning of the actual surgical procedure will not pose any additional risks in participation. The small amount of blood drawn by the anesthesiologist for the purposes of the research study will have no impact on your surgical procedure.

Option B
The small amount of blood drawn by the phlebotomist for the purposes of the research study may cause you some discomfort. The blood will be taken from a vein in your arm. This may cause temporary discomfort or a bruise at the site of the needle puncture.

The molecular and genetic analyses that will be conducted on tissue specimens may discover known or unknown genetic alterations. The results of these analyses will not be released to you or your personal care provider. These tests are experimental and are of unknown value in the diagnosis and treatment of ovarian cancer. However, this non-identified information may be shared with investigators at other, additional, institutions. Not knowing these results may be of potential discomfort to you or may not be of concern to you.

BENEFITS

There is no direct benefit to you for participating in this study. The knowledge obtained from studying the specimens may help with earlier detection and prevention of ovarian cancer, identify the causes of ovarian cancer and/or lead to better treatment for ovarian cancer.

ALTERNATIVES

The alternative to participating in this study is not to have tissue and blood provided to the investigators in this research study.

USE OF SPECIMENS

Histopathologic material (tissue specimens) and blood which is stored at the study repository may be made available to researchers at the participating institutions and other

institutions for future research studies which could result in the development of commercially useful products.

YOUR PARTICIPATION

Your participation in this study is *voluntary*. Once enrolled, you may discontinue participation at any time for any reason, without notice.

Declining to participate in this study will involve no penalty or loss of benefits to which you may otherwise be entitled. You will continue to receive your usual medical care even if you decide not to participate in this study. Questions about this study may be addressed to the Principal Investigator, your surgeon, or research staff.

COSTS AND COMPENSATION

It should be understood that whether or not you participate, all medical expenses relating to your surgical procedures will be paid by you and/or your insurance company. There are no additional costs to you for participating in this study.

The Department of Defense (USAMRMC) is funding this research project. Should you be injured as a direct result of participating in this research project, you will be provided medical care, at no cost to you, for that injury. You will not receive any injury compensation, only medical care. You should also understand that this is not a waiver or release of your legal rights. You should discuss this issue thoroughly with the Principal Investigator before you enroll in this study.

There is no financial compensation for participation in this program. By agreeing to participate in this research study, you understand that you waive any claim to monetary gain or financial benefit as relates to this study. If you have any questions regarding your costs, financial responsibilities, and/or medical insurance coverage for this activity, please contact your primary care physician or Pacific Gynecology Specialists at (206) 587-0585.

CONFIDENTIALITY OF RECORDS

We have taken extensive precautions to maintain the confidentiality of all study records. All records containing personal information will be kept confidential as provided by law. Strict protocols will be followed to maintain the confidentiality of any identified patient information. These procedures include a pledge of confidentiality by all study personnel at Fred Hutchinson Cancer Research Center, Pacific Gynecology Specialists and the University of Washington, data handling procedures, network and password protection, and proper storage and handling of all files and specimens. Study records will be maintained indefinitely for the purpose of analysis and follow up.

Your personal identity will not be revealed in any publication or release of results. Please be aware that representatives from the U.S. Army Medical Research and Materiel

Command will also have access to the study records, and may inspect the records of the research in their duty to protect human subjects in research.

OTHER INFORMATION

Your participation is critical to the success of this study. We want you to know that you are the most important part of this study. Without your participation, this type of study will not be possible.

We have tried to make participation as convenient and easy as possible for you. Please let us know if there are ways you think it would be easier for you and others to participate in this study.

If you have any questions about the research study, or in the event of a research-related injury, please contact the Principal Investigator, Nicole Urban in the Division of Public Health Sciences at Fred Hutchinson Cancer Research Center at (206) 667-4677.

If you have any questions specifically about your rights as a research participant, please contact Karen Hansen in the Institutional Review Office of the Fred Hutchinson Cancer Research Center at (206) 667-4867.

Investigator's Statement

I have furnished a qualified and trained study nurse or research staff member to provide an explanation of the above research program. The patient was given an opportunity to discuss the procedures, including possible alternatives, with this person or their physician and to ask any additional questions. In addition the patient was provided with my name and telephone number and informed that she could contact me for any additional questions. A signed copy of the consent form has been given to the patient.

Nicole Urban June 8, 1999

Investigator's Signature Date

Patient's Statement:

I agree to this study and to the conditions outlined in this consent form as relates to:

- ☐ **Option A (Ovarian tissue and blood samples)**

- ☐ **Option B (Blood samples only)**

Please check relevant box above.

I have had the opportunity to ask questions about the study and my participation and about the need for access to my medical records. They have been answered to my satisfaction. I understand future questions I may have about the research will be answered by one of the investigators listed above and that any questions I have about my rights as a research participant will be answered by the person identified above. I give permission for my medical records to be available for review and copying to the appropriate physicians and personnel for this study at the Fred Hutchinson Cancer Research Center, Pacific Gynecology Specialists, University of Washington and United States Army Medical Research and Materiel Command. I understand that there is a possibility that the blood, tissue and bodily fluids (specimens) which I am providing under this study may also be used in other research studies and could potentially have some commercial applicability.

____
Patient's Name (printed) Date

____
Patient's Signature Date

Patient's Permanent Address:

____

____

____

____
Witness Name (printed) Date

____
Witness Signature Date

Copies to: Patient
Medical Records
Research File

Patient's Statement of Donation:

I voluntarily and freely donate any and all blood, tissues and bodily fluid to the Fred Hutchinson Cancer Research Center and hereby relinquish all right, title, and interest to said items.

____

Patient's Name (printed) Date

____

Patient's Signature Date

____

Witness Name (printed) Date

____

Witness Signature Date

Copies to:

Patient
Medical Records
Research File

ORCHID

Participant Enrollment Form & Medical Records Release

Name: ____
LAST FIRST M.I.

Date of Birth: ____/____/____ **Social Security Number:** ____-____-____

Permament Address: ____
STREET ADDRESS APT.

____
CITY STATE ZIP

Home Phone: (____) ____-________ **Work Phone:** (____) ____-________

Next of Kin Contact:
(A friend or relative who does not live with you and who could provide information should you move.)

Name: ______________________________
Relationship: ______________________________
Address: ______________________________
____
Home Phone: (____) ____-________

I hereby authorize Dr. ____________________ **to disclose the following information from my medical records to:** Investigators and staff of the ORCHID study
Nicole Urban, ScD (Principal Investigator)
1100 Fairview Ave., N. MP-804
PO Box 19024
Seattle, WA 98109 (206) 667-4677

Information to be disclosed:

_X_ Doctors Pertinent _X_ Operative Report _X_ History & Physical Examination
_X_ Test Results *(Please Check)*

☐ EKG ☐ LAB ☐ PATHOLOGY ☐ X-RAY ☐ OTHER: ____________

For the purpose of: Research

I understand that this authorization, unless expressly limited by me in writing, will extend to all aspects of treatment, including testing and/or treatment for Sexually Transmitted Diseases, AIDS or HIV Infection, Alcohol and/or Drug Abuse or Mental Health Conditions.

I understand that this authorization may be revoked in writing at any time, except to the extent that action has been taken in reliance on this authorization. Unless otherwise revoked, this authorization expires 90 days from the date signed below.

The faculty, its employees, officers and physicians are hereby released from any legal responsibility of liability for disclosure of the above information to the extent indicated and authorized herein.

Signed: ______________________________ Date: ____/____/____
(PATIENT)

______________________________ Date: ____/____/____
(OR LEGAL REPRESENTATIVE AND RELATIONSHIP)

Fred Hutchinson Cancer Research Center
1100 Fairview Avenue North Seattle, Washington 98109-1024 (206) 667-5000

FOR STAFF USE ONLY	Inst: ☐ PGS ☐ PMC ☐ PRV ☐ UW		Packet no.:
Med. Rec. ID: ________	Physician ID: ________	Enrolled by: ________	

ORCHID

How to Fill Out this Questionnaire

This questionnaire asks you about your general health, medical history, family history, and some lifestyle habits. It takes about 20 minutes to complete. All of your answers will be kept strictly confidential.

Before you begin, please note the following.

1. If a question asks you to "check one" answer, please check the one that best describes you.
2. If you are unsure about how to answer a question, make your best guess. If you cannot provide a guess or estimate, please check or write "Don't know".
3. Some questions ask about "full" and "half" brothers and sisters. A "full" brother or sister has the same two parents as you. A "half" brother or sister has only one of the same parents as you.

Thank you for taking the time to complete this questionnaire. Your participation is greatly appreciated!

QUESTIONS 1-6

1. What is today's date?

   Month | Day | Year

2. What is your date of birth?

   Month | Day | Year

3. In what country were you born?

   ____

4. How tall are you?

   Feet | Inches

5. How much did you weigh one year ago?

   Pounds

6. What is your marital status now?

   - ☐1 Married or living as married
   - ☐2 Widowed
   - ☐3 Divorced
   - ☐4 Separated
   - ☐5 Never Married

QUESTIONS 7-23 ARE ABOUT YOUR REPRODUCTIVE AND MEDICAL HISTORY.

7. How old were you when you had your first menstrual period?

   |__|__| Years old

8. During most of your life, were your periods regular; that is, did they occur about once a month? **(Do not include any times when you were pregnant or taking birth control pills.)**

   ❑ 1 No ❑ 2 Yes ❑ 3 Sometimes regular, sometimes irregular

   8.1 How old were you when your periods first became regular? **(Your __best__ guess.)**

   |__|__| Years old

9. Between the time you had your first period and your last period, did you ever go without any periods for at least one year? **(Do not count times when you were pregnant or breastfeeding.)**

   ❑ 1 No ❑ 2 Yes

   9.1 Between your first menstrual period and your last, all together, about how long did you go without having your period? **(Again, do not count times when you were pregnant or breastfeeding.)**

   |__|__| Months

Go to the next page.

10. Have you ever been pregnant?

☐1 No ☐2 Yes ☐8 Don't know → **(Go to Question 11)**

10.1 Including live births, stillbirths, miscarriages, abortions, tubal, or ectopic pregnancies, how many times have you been pregnant? **(If you are currently pregnant, be sure to count this pregnancy.)**

[|] Number of pregnancies

10.2 How many of your pregnancies lasted 6 or more months?

[|] Number of pregnancies

10.3 How many live births resulted from these pregnancies?

[|] Number of live births

10.4 How old were you when you had your first live birth or stillbirth?

[|] Years old

11. Have you ever tried to become pregnant for more than 1 year without becoming pregnant?

☐1 No ☐2 Yes ☐8 Don't know

11.1 Did you visit a doctor or clinic because you didn't get pregnant?

☐1 No ☐2 Yes

11.2 Was a reason found for why you did not become pregnant?

☐1 No ☐2 Yes
☐8 Don't know

11.3 What was the reason you did not become pregnant? **(Mark all that apply)**

☐1 Problem with your hormones or ovulation (Producing eggs)
☐2 Problem with your tubes or uterus
☐3 Endometriosis
☐4 Other problem with you (Specify): ________________

____

☐5 Problem with partner
☐8 Don't know

Go to the next page.

12. Did you ever breastfeed or nurse any children for at least one month?

☐₁ No ☐₂ Yes

12.1 How many children did you breastfeed?	⎣ ⋮ ⎦	Number of children
12.2 How old were you when you first breastfed a child?	⎣ ⋮ ⎦	Years old
12.3 How old were you when you last breastfed a child?	⎣ ⋮ ⎦	Years old
12.4 Thinking about all the children you breastfed, how many months total did you breastfeed? **(Your best guess)**	⎣ ⋮ ⎦	Number of months

13. Have you ever used birth control pills for any reason?

☐₁ No ☐₂ Yes ☐₈ Don't know ➜ **(Go to Question 14)**

13.1 How old were you when you first used birth control pills?	⎣ ⋮ ⎦	Years old
13.2 How many years altogether have you used birth control pills?	⎣ ⋮ ⎦	Number of years

14. Did you ever have an operation to have your tubes tied to prevent pregnancy?

☐₁ No ☐₂ Yes

14.1 How old were you when you had your tubes tied?

⎣ ⋮ ⎦ Years old

Go to the next page.

15. Have you had a menstrual period in the last 12 months?

☐1 No ☐2 Yes ☐8 Don't know

16. When was your last menstrual period (best guess)? [|] Month [|] Year

17. How would you describe your current menstrual periods? Mark the one statement that best describes your situation.

- ☐1 Still having periods, or currently pregnant or nursing
- ☐2 Possibly going through menopause (the change of life)
- ☐3 Periods stopped by themselves (natural menopause)
- ☐4 Periods stopped by surgery (removal of uterus, ovaries, or both)
- ☐5 Still having periods due to hormone replacement therapy
- ☐8 Other (Specify): ______________________

____

18. Have you had a hysterectomy (surgical removal of your uterus)?

☐1 No ☐2 Yes ☐8 Don't know → **(Go to Question 19)**

18.1 How old were you when you had a hysterectomy?

[|] Years old

19. Have you ever used estrogen pills, creams, or skin patches (for example, Premarin, Estrace, Ogen, Estraderm)? Sometimes estrogens are given to treat symptoms of menopause (e.g. hot flashes, night sweats), to prevent osteoporosis (thin or brittle bones), or to prevent heart disease. **(Include all hormones except pills used for birth control.)**

☐1 No ☐2 Yes ☐8 Don't know → **(Go to Question 20)**

19.1 How old were you when you first used estrogen? [|] Years old

19.2 How many years altogether have you used estrogen? [|] Number of years

19.3 When did you last use estrogen? [|] Month [|] Year

Go to the next page.

20. Did a doctor ever say that you had any of the following conditions?

20.1	Diabetes, high blood sugar, or sugar diabetes	❑1 No	❑2 Yes	❑8 Don't know
20.2	Inflammatory bowel disease, colitis, or Crohn's disease	❑1 No	❑2 Yes	❑8 Don't know
20.3	Chronic lung disease, bronchitis, or emphysema	❑1 No	❑2 Yes	❑8 Don't know
20.4	Heart failure or congestive heart failure	❑1 No	❑2 Yes	❑8 Don't know
20.5	Heart attack, coronary, or myocardial infarction	❑1 No	❑2 Yes	❑8 Don't know
20.6	High blood cholesterol requiring pills	❑1 No	❑2 Yes	❑8 Don't know
20.7	High blood pressure (hypertension)	❑1 No	❑2 Yes	❑8 Don't know
20.8	Stroke or brain hemorrhage	❑1 No	❑2 Yes	❑8 Don't know
20.9	Liver disease or cirrhosis	❑1 No	❑2 Yes	❑8 Don't know
20.10	Chronic kidney disease or kidney failure	❑1 No	❑2 Yes	❑8 Don't know
20.11	Depression or anxiety requiring pills	❑1 No	❑2 Yes	❑8 Don't know
20.12	Osteoporosis (weak, thin, or brittle bones)	❑1 No	❑2 Yes	❑8 Don't know
20.13	Asthma	❑1 No	❑2 Yes	❑8 Don't know
20.14	Thyroid problem (not cancer)	❑1 No	❑2 Yes	❑8 Don't know
20.15	Fibroids (benign tumors) in your uterus or womb	❑1 No	❑2 Yes	❑8 Don't know
20.16	Endometriosis	❑1 No	❑2 Yes	❑8 Don't know
20.17	Benign breast disease or fibrocystic breast disease	❑1 No	❑2 Yes	❑8 Don't know
20.18	Pelvic inflammatory disease, PID or pelvic infection	❑1 No	❑2 Yes	❑8 Don't know
20.19	Rheumatoid arthritis, SLE or scleroderma	❑1 No	❑2 Yes	❑8 Don't know
20.20	Polycystic ovarian disease, PCO or sclerocytic ovaries	❑1 No	❑2 Yes	❑8 Don't know
20.21	Ovarian cyst	❑1 No	❑2 Yes	❑8 Don't know

21. Did a doctor ever say that you had breast cancer?

☐1 No ☐2 Yes ☐8 Don't know → **(Go to Question 22)**

21.1 How old were you when you were first diagnosed with breast cancer? [|] Years old

21.2 Was the breast cancer found in one or both breasts?

One	Both	Don't know
☐1	☐2	☐8

22. Did a doctor ever say that you had ovarian cancer?

☐1 No ☐2 Yes ☐8 Don't know → **(Go to Question 23)**

22.1 How old were you when you were first diagnosed with ovarian cancer? [|] Years old

23. Did a doctor ever say that you had any other type of cancer?

☐1 No ☐2 Yes ☐8 Don't know → **(Go to Question 24)**

23.1 What type of cancer did you have?	How old were you when you were first diagnosed with this cancer?
______________________	[|] Years old
______________________	[|] Years old
______________________	[|] Years old

Go to the next page.

QUESTIONS 24-33 ARE ABOUT YOUR FAMILY.

24. Are you adopted?

☐1 No ☐2 Yes ☐8 Don't know

25. Are you a twin?

☐1 No ☐2 Yes ☐8 Don't know → **(Go to Question 26)**

25.1 Are you an identical or fraternal twin?

Identical	Fraternal	Don't know
☐1	☐2	☐8

Go to the next page.

26. Please complete the following questions on **female** family members related to you by blood (that is, not related to you by marriage or by adoption). ***Include all blood relatives, both those living and deceased.*** For each relative type, write the number in your family. **(Write "00" in the box if the answer is "none", and "88" if the answer is "Don't know".)**

26.1 How many full sisters do you have?	☐☐	Full sisters
26.2 How many half sisters do you have?	☐☐	Half sisters
26.3 How many daughters do you have, not including step daughters?	☐☐	Daughters
26.4 How many full sisters does your mother have?	☐☐	Aunts
26.5 How many half sisters does your mother have?	☐☐	Aunts
26.6 How many full sisters does your father have?	☐☐	Aunts
26.7 How many half sisters does your father have?	☐☐	Aunts
26.8 How many nieces do you have?	☐☐	Nieces

27. Have any of the following female relatives related to you by blood ever been diagnosed with breast cancer?

27.1 Mother	☐$_1$ No	☐$_2$ Yes	☐$_8$ Don't know
27.2 Grandmother(s)	☐$_1$ No	☐$_2$ Yes → How many? ☐☐	☐$_8$ Don't know
27.3 Full sister(s)	☐$_1$ No	☐$_2$ Yes → How many? ☐☐	☐$_8$ Don't know
27.4 Half sister(s)	☐$_1$ No	☐$_2$ Yes → How many? ☐☐	☐$_8$ Don't know
27.5 Daughter(s)	☐$_1$ No	☐$_2$ Yes → How many? ☐☐	☐$_8$ Don't know
27.6 Aunt(s) on father's side	☐$_1$ No	☐$_2$ Yes → How many? ☐☐	☐$_8$ Don't know
27.7 Aunt(s) on mother's side	☐$_1$ No	☐$_2$ Yes → How many? ☐☐	☐$_8$ Don't know
27.8 Niece(s)	☐$_1$ No	☐$_2$ Yes → How many? ☐☐	☐$_8$ Don't know

28. Have any of the following female relatives related to you by blood ever been diagnosed with ovarian cancer?

28.1 Mother	❑1 No	❑2 Yes	❑8 Don't know
28.2 Grandmother(s)	❑1 No	❑2 Yes → How many? [:]	❑8 Don't know
28.3 Full sister(s)	❑1 No	❑2 Yes → How many? [:]	❑8 Don't know
28.4 Half sister(s)	❑1 No	❑2 Yes → How many? [:]	❑8 Don't know
28.5 Daughter(s)	❑1 No	❑2 Yes → How many? [:]	❑8 Don't know
28.6 Aunt(s) on father's side	❑1 No	❑2 Yes → How many? [:]	❑8 Don't know
28.7 Aunt(s) on mother's side	❑1 No	❑2 Yes → How many? [:]	❑8 Don't know
28.8 Niece(s)	❑1 No	❑2 Yes → How many? [:]	❑8 Don't know

29. Please complete the following questions on **male** family members related to you by blood. ***Include all blood relatives, both those living and deceased***. For each relative type, write the number in your family. **(Write "00" in the box if the answer is "none", and "88" if the answer is "Don't know".)**

29.1 How many full brothers do you have?	[:]	Full brothers
29.2 How many half brothers do you have?	[:]	Half brothers
29.3 How many sons do you have, not including step sons?	[:]	Sons
29.4 How many full brothers does your mother have?	[:]	Uncles
29.5 How many half brothers does your mother have?	[:]	Uncles
29.6 How many full brothers does your father have?	[:]	Uncles
29.7 How many half brothers does your father have?	[:]	Uncles
29.8 How many nephews do you have?	[:]	Nephews

30. Have any of the following male relatives related to you by blood ever been diagnosed with prostate cancer?

30.1 Father	☐1 No	☐2 Yes	☐8 Don't know
30.2 Grandfather(s)	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
30.3 Full brother(s)	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
30.4 Half brother(s)	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
30.5 Son(s)	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
30.6 Uncle(s) on father's side	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
30.7 Uncle(s) on mother's side	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
30.8 Nephew(s)	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know

31. Have any of the following male relatives related to you by blood ever been diagnosed with breast cancer?

31.1 Father	☐1 No	☐2 Yes	☐8 Don't know
31.2 Grandfather(s)	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
31.3 Full brother(s)	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
31.4 Half brother(s)	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
31.5 Son(s)	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
31.6 Uncle(s) on father's side	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
31.7 Uncle(s) on mother's side	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know
31.8 Nephew(s)	☐1 No	☐2 Yes → How many? ☐☐	☐8 Don't know

32. Have any other types of cancer or malignant tumors occurred in any of the family members (blood relatives) you listed in questions 26 and 29?

☐$_1$ No ☐$_2$ Yes ☐$_8$ Don't know → **(Go to Question 33)**

32.1 What other type(s) of malignant tumors? (Check all that apply and list how many relatives had each type.)

- ☐ Bladder — How many? ☐☐
- ☐ Brain — How many? ☐☐
- ☐ Colon — How many? ☐☐
- ☐ Kidney — How many? ☐☐
- ☐ Leukemia — How many? ☐☐
- ☐ Lung — How many? ☐☐
- ☐ Lymphoma — How many? ☐☐
- ☐ Melanoma — How many? ☐☐
- ☐ Pancreas — How many? ☐☐
- ☐ Skin (not melanoma) — How many? ☐☐
- ☐ Thyroid — How many? ☐☐
- ☐ Uterus, womb, or endometrium — How many? ☐☐
- ☐ Cervix — How many? ☐☐
- ☐ Other cancer(s) or malignant tumors (Specify): ________________ — How many? ☐☐

33. Do any other types of health problems, disorders or diseases tend to run in your family (in two or more blood relatives)?

☐$_1$ No ☐$_2$ Yes ☐$_8$ Don't know → **(Go to Question 34)**

33.1 What is the problem or disease?	How many members of your family have this problem or disease?
________________	☐☐ Relatives
________________	☐☐ Relatives

Go to the next page.

QUESTIONS 34-35 ASK ABOUT YOUR LIFESTYLE HABITS.

34. Have you smoked a total of 100 cigarettes or more in your lifetime?

❑1 No ❑2 Yes ❑8 Don't know → **(Go to Question 35)**

34.1 How old were you when you first started smoking cigarettes? [|] Years old

34.2 Do you smoke cigarettes now?
❑1 No ❑2 Yes → **(Go to Question 34.4)**

34.3 How old were you when you last stopped smoking cigarettes?
[|] Years old → **(Go to Question 34.5)**

34.4 How many cigarettes <u>each day</u> do you smoke? [|] Per day

34.5 How many <u>total</u> years have you smoked (or did you smoke)? [|] Years

35. Did you ever drink alcoholic beverages (beer, wine, or liquor) at least once a month for 6 months or more?

❑1 No ❑2 Yes ❑8 Don't know → **(Go to Question 36)**

35.1 Have you had any alcoholic beverages during the past six months?

❑1 No ❑2 Yes ❑8 Don't know → **(Go to Question 36)**

35.2. During the past <u>six months</u>, how many alcoholic beverages did you usually have?

- ❑1 None or less than <u>one</u> per month
- ❑2 1-3 each month
- ❑3 4-6 each month
- ❑4 1 each day
- ❑5 2-4 each day
- ❑6 5 or more each day

Go to the next page.

QUESTIONS 36-38 ASK ABOUT YOUR BACKGROUND

36. What is the highest level of education that you have completed? **(Check one)**

- ❑1 8th grade or less
- ❑2 9th - 11th grade
- ❑3 Graduated from high school (or GED)
- ❑4 Vocation, technical, or business training
- ❑5 Some college or junior college
- ❑6 Graduated from college
- ❑7 Attended graduate or professional school

37. How would you describe your racial or ethnic group? If you are of mixed descent, with which group do you identify most? **(Check one)**

- ❑1 Native American, American Indian, or Alaska Native
- ❑2 Asian or Pacific Islander
- ❑3 Black or African American (not of Hispanic origin)
- ❑4 Hispanic/Latino (ancestry is Mexican, Cuban, Puerto Rican, Central American, or South American)
- ❑5 White (not of Hispanic origin)
- ❑8 Other (Specify):________________________________

38. What was your job status one year ago? Check the one that best describes you.

- ❑01 Working full-time (35 hours per week or more)
- ❑02 Working part-time
- ❑03 On temporary leave
- ❑04 Not working for pay
- ❑05 Retired
- ❑06 Disabled, unable to work
- ❑07 In school, not working for pay
- ❑08 Homemaker, raising children, care of others
- ❑10 Other (Specify): ________________________________

QUESTIONS 39-49 ASK ABOUT FEELINGS YOU MAY HAVE ABOUT POSSIBLE DIFFICULTIES IN YOUR LIFE. SOME OF THEM ALSO ASK ABOUT YOUR SOURCES OF SOCIAL SUPPORT FOR DEALING WITH THOSE DIFFICULTIES.

In the last month, how often have you: **(Check one box on each line.)**

	Never	Almost never	Sometimes	Fairly often	Very often
39. Felt you were unable to control important things in your life?	❑1	❑2	❑3	❑4	❑5
40. Felt confident about your ability to handle personal problems?	❑1	❑2	❑3	❑4	❑5
41. Felt things were going your way?	❑1	❑2	❑3	❑4	❑5
42. Felt difficulties were piling up so high that you could not overcome them?	❑1	❑2	❑3	❑4	❑5

People sometimes look to others for help, friendship, or other types of support. Next are some questions about the support that you have. How often is each of the following kinds of support available to you if you need it? **(Check one box on each line.)**

	None of the time	A little of the time	Some of the time	Most of the time	All of the time
43. Someone you can count on to listen to you when you need to talk.	❑1	❑2	❑3	❑4	❑5
44. Someone to give you good advice about a problem.	❑1	❑2	❑3	❑4	❑5
45. Someone to take you to the doctor if you need it.	❑1	❑2	❑3	❑4	❑5
46. Someone to help with daily chores if you are sick.	❑1	❑2	❑3	❑4	❑5
47. Someone to share your most private worries and fears.	❑1	❑2	❑3	❑4	❑5
48. Someone to do something fun with.	❑1	❑2	❑3	❑4	❑5
49. Someone to love you and make you feel wanted.	❑1	❑2	❑3	❑4	❑5

LABEL

Interest in Future Research Studies

Your participation in this research study has been very important. If you would like to be contacted about participating in future research studies on cancer and its prevention, please indicate so by checking the appropriate box and completing the following information:

☐$_{01}$ I am interested in being contacted about future research studies.

☐$_{02}$ I am *not* interested in being contacted about future research studies.

Your signature: ______________________________

Name (please print): ______________________________
First name *Last name*

Address: ______________________________
Street *Apt. #*

____
City *State* *Zip*

Telephone: (   ) ____________________

If you indicate an interest in participating in future studies, your name will be kept for five (5) years so that researchers may contact you when a study is initiated. Agreeing to be contacted does not mean that you have to participate in a future research study – only that you will consider it.

Thank you again for your cooperation.

Thank you very much for completing this questionnaire.

Appendix C

Core Specimen Collection and Transport Forms

Statistical, Clinical and Laboratory Coordinating Core

Nicole Urban, ScD, Garnet Anderson, PhD, Nancy Kiviat, MD,
Leona Holmberg, MD, Jane Kuypers, PhD,
Charles Drescher, MD, Mary Anne Rossing, PhD

ORCHID - *Specimen Collection Form*

THIS FORM MUST ACCOMPANY ALL PATIENT SPECIMENS SUBMITTED TO THE CORE LABORATORY AND REPOSITORY

UPN: ________ Procedure: ______________ Surgeon ID: ________ Procedure date: ___/___/___

Patient name: ________________ (LAST, FIRST, MI) Institution of Proc.: ☐ SMC ☐ UW ☐ PRV TCS ID: ________

Informed Consent Verified? ☐ No ☐ Yes Case status: ☐ Incident ☐ Recurrent Chemo prior to surgery? ☐ No ☐ Yes

Specimen Information Place the duplicate barcode in the appropriate section (as applicable) and circle side from which each was removed.

	Primary	*Ovary*	*Contralateral*	*Ovary*	*Metastatic*	*Site*
STM		R L	**Comments/Notes:**			
Form. OCT		R L				
Formalin		R L		R L		
Frozen		R L		R L		
		R L		R L		
		R L		R L		
		R L		R L		
		R L		R L		
		R L		R L		
		R L		R L		
Total: ____			Total: ____		Total: ____	

Total frozen ____

Blood

10 ml red tops: ________

5 ml EDTA tubes: ________

Time of collection: __:__ AM / PM

WBC pellets

Plasma

ORCHID – *Blood Specimen Form*

UPN: ____________ Patient name: ______________________________
LAST FIRST MI

DynaCare requisition no.: ______________ Blood drawn in: ❑ surgery ❑ clinic

Date of blood draw: ____/____/____

Processing & Transport

❑ No. tubes submitted for processing: ☐ Red top ☐ EDTA

❑ Transported to freezer holding area after processing ________
INITIALS

In the spaces below, record the 6-digit number on the label of each vial received from the processing labs. Please note if any vial is only partially full, or if the contents are hemolyzed or lipidemic.

Serum

WBC Pellets

Plasma

ORCHID – *Specimen Tracking Form*

Date: ______/______/______

Specimens sent from: ORCHID Core Repository

- ❑ -20° C freezer
- ❑ -70° C freezer
- ❑ liquid nitrogen freezer

Specimen type	**Number**
❑ Tissue	______
❑ Serum	______
❑ Other	______

Time of packaging: ____:______ AM / PM

Transported on:
- ❑ dry ice
- ❑ liquid nitrogen

Specimens packaged for delivery:

Specimen no.	T	S	O	note	Specimen no.	T	S	O	note
________	❑	❑	❑	________	________	❑	❑	❑	________
________	❑	❑	❑	________	________	❑	❑	❑	________
________	❑	❑	❑	________	________	❑	❑	❑	________
________	❑	❑	❑	________	________	❑	❑	❑	________
________	❑	❑	❑	________	________	❑	❑	❑	________
________	❑	❑	❑	________	________	❑	❑	❑	________
________	❑	❑	❑	________	________	❑	❑	❑	________
________	❑	❑	❑	________	________	❑	❑	❑	________
________	❑	❑	❑	________	________	❑	❑	❑	________
________	❑	❑	❑	________	________	❑	❑	❑	________

Specimens received:

Receiving laboratory:
- ❑ Kiviat laboratory
- ❑ M. Schummer laboratory (UW)
- ❑ B. Nelson laboratory (VMMC)
- ❑ N. Disis laboratory (UW)
- ❑ Hellstrom laboratory (PNRF)
- ❑ Univ. of Washington: ________
- ❑ FHCRC: ____________

Time of delivery receipt: ____:_____ AM / PM

Date of receipt: ______/______/______

- ❑ Contents above confirmed
- ❑ Contents different as noted: ______________________________

Investigator/lab personnel signature: ______________________________

TCS initials: ____________

Appendix D

Core Clinical Data Collection Forms

Statistical, Clinical and Laboratory Coordinating Core

Nicole Urban, ScD, Garnet Anderson, PhD, Nancy Kiviat, MD,
Leona Holmberg, MD, Jane Kuypers, PhD,
Charles Drescher, MD, Mary Anne Rossing, PhD

ORCHID – *Specimen Histology Report I*

UPN: ________________

Date of analysis: ____/____/____
Form completed by: ______________

Clinical diagnosis: ______________________________

Site of Primary Ca.: ☐ Ovarian
☐ Other:

Pathology Diagnosis:

☐ **Normal**

☐$_{03}$ Normal ovarian/tubal tissue
☐$_{05}$ Other normal:

☐ **Malignant**

Serous
☐$_{16}$ Serous carcinoma of LMP
☐$_{17}$ Serous carcinoma
☐$_{19}$ Serous cystadenofibroma
☐$_{20}$ Serous adenofibroma

Mucinous
☐$_{28}$ Mucinous adenocarcinoma of LMP
☐$_{29}$ Mucinous carcinoma
☐$_{30}$ Malignant mucinous adenofibroma
☐$_{31}$ Malignant mucinous cystadenofibroma

☐$_{57}$ Other:

Endometrioid
☐$_{37}$ Endometrioid carcinoma of LMP
☐$_{38}$ Endometrioid adenocarcinoma

Other
☐$_{40}$ Malignant adenosarcoma (mesodermal)
☐$_{41}$ Mesodermal (mullerian) mixed tumor, homo.
☐$_{42}$ Mesodermal (mullerian) mixed tumor, hetero.
☐$_{47}$ Clear cell carcinoma of LMP
☐$_{48}$ Clear cell carcinoma
☐$_{52}$ Brenner tumor of LMP
☐$_{53}$ Malignant Brenner tumor
☐$_{54}$ Undifferentiated carcinoma
☐$_{55}$ Adenocarcinoma, NOS

☐ **Benign**

Serous
☐$_{11}$ Serous cystadenoma
☐$_{12}$ Serous adenofibroma
☐$_{13}$ Serous cystadenofibroma
☐$_{14}$ Proliferating serous adenofibroma
☐$_{15}$ Proliferating serous cystadenofibroma

Mucinous
☐$_{25}$ Mucinous cystadenoma
☐$_{26}$ Mucinous adenofibroma
☐$_{27}$ Mucinous cystadenofibroma

Non-neoplastic
☐$_{06}$ Paraovarian cyst
☐$_{07}$ Functional cyst
☐$_{08}$ Corpus luteum
☐$_{09}$ Inflammatory lesion
☐$_{10}$ Endometriosis

Endometrioid
☐$_{32}$ Endometrioid cystadenoma
☐$_{33}$ Endometrioid adenofibroma
☐$_{34}$ Endometrioid cystadenofibroma
☐$_{35}$ Proliferating endometrioid adenofibroma
☐$_{36}$ Proliferating endometrioid cystadenofibroma

Other
☐$_{39}$ Benign adenofibroma (mesodermal)
☐$_{43}$ Clear cell adenofibroma
☐$_{44}$ Clear cell cystadenofibroma
☐$_{45}$ Proliferating clear cell adenofibroma
☐$_{46}$ Proliferating clear cell cystadenofibroma
☐$_{49}$ Benign Brenner tumor, typical
☐$_{50}$ Metaplastic Brenner tumor
☐$_{51}$ Proliferating Brenner tumor

☐$_{98}$ Other:

Tumor Grade: ☐$_a$ well differentiated ☐$_b$ moderately differentiated ☐$_c$ poorly differentiated

FIGO Stage:

☐$_{01}$ IA	☐$_{04}$ IIA	☐$_{07}$ IIIA	☐$_{10}$ IVA
☐$_{02}$ IB	☐$_{05}$ IIB	☐$_{08}$ IIIB	☐$_{11}$ IVB
☐$_{03}$ IC	☐$_{06}$ IIC	☐$_{09}$ IIIC	☐$_{12}$ IVC

ORCHID – *Specimen Histology Report II*

Patient ID: ____________

Date of analysis: ____/____/____
Form completed by: ____________

A Site: ______
Path. dx.: ______
Necrosis: ______ %
Normal cells: ______ %

B Site: ______
Path. dx.: ______
Necrosis: ______ %
Normal cells: ______ %

C Site: ______
Path. dx.: ______
Necrosis: ______ %
Normal cells: ______ %

D Site: ______
Path. dx.: ______
Necrosis: ______ %
Normal cells: ______ %

Site

1 Primary ovarian tumor
2 Contralateral ovary – NL
3 Metastatic tumor
4 Non-ovarian tissue – NL
5 Not known
6 Ovarian tissue – NL
7 Tube – NL
8 Uterus
9 Other (specify)

Differentiation

a well differentiated
b moderately differentiated
c poorly differentiated

Pathology Diagnosis

Non-neoplastic lesions

1 Inadequate
2 Necrosis only
3 Normal ovarian or tubal tissue
4 Normal fibrovascular tissue
5 Normal other (specify)
6 Benign cyst/paraovarian
7 Functional cyst
8 Corpus luteum
9 Inflammatory lesion
10 Endometriosis

Epithelial Tumors

Serous tumors, benign
11 Serous cystadenoma
12 Serous adenofibroma
13 Serous cystadenofibroma
14 Proliferating serous adenofibroma
15 Proliferating serous cystadenofibroma

Serous tumors, malignant
16 Serous carcinoma of LMP
17 Serous carcinoma
19 Serous cystadenofibroma
20 Serous adenofibroma

Mucinous tumors, benign
25 Cystadenoma
26 Adenofibroma
27 Cystadenofibroma

Mucinous tumors, malignant
28 Mucinous adenocarcinoma of LMP
29 Mucinous carcinoma
30 Malignant mucinous adenofibroma
31 Malignant mucinous cystadenofibroma

Endometrioid tumors, benign
32 Endometrioid cystadenoma
33 Endometrioid adenofibroma
34 Endometrioid cystadenofibroma
35 Proliferating endometrioid adenofibroma
36 Proliferating endometrioid cystadenofibroma

Endometrioid tumors, malignant
37 Endometrioid carcinoma of LMP
38 Endometrioid adenocarcinoma

Mesodermal mixed tumors
39 Benign adenofibroma
40 Malignant adenosarcoma
41 Mesodermal (mullerian) mixed tumor, homo.
42 Mesodermal (mullerian) mixed tumor, hetero.

Clear cell tumors, benign
43 Clear cell adenofibroma
44 Clear cell cystadenofibroma
45 Proliferating clear cell adenofibroma
46 Proliferating clear cell cystadenofibroma

Clear cell tumors, malignant
47 Clear cell carcinoma of LMP
48 Clear cell carcinoma

Brenner tumors, benign
49 Benign Brenner tumor, typical
50 Metaplastic Brenner tumor
51 Proliferating Brenner tumor

Brenner tumors, malignant
52 Brenner tumor of LMP
53 Malignant Brenner tumor

Other
54 Undifferentiated carcinoma
55 Adenocarcinoma, NOS
56 Unclassified epithelial tumor
57 Neoplastic other (specify)

98 Non-neoplastic other (specify)
99 Other (specify)

ORCHID – *Clinical Data Form*

This form should be completed 1 to 2 weeks following a participant's surgery; this allows time for all surgical and pathology reports to be submitted to her medical records file.

UPN: ____________________ Form completed by: ____________________

Name: ____________________ Physician ID: ____________________

Med. records ID: ____________________ Location of records: ☐1 PGS ☐2 UW Gyn. Onc.

I. Presenting symptoms & duration

☐ H&P not in clinic records

Symptom	Report of symptom during history		Symptom duration (weeks) <4	4-8	>8	Not noted in H & P
1. Pain	☐1 Neg.	☐2 Pos. → Duration?	☐1	☐2	☐3	☐8
2. Distention	☐1 Neg.	☐2 Pos. → Duration?	☐1	☐2	☐3	☐8
3. Bleeding	☐1 Neg.	☐2 Pos. → Duration?	☐1	☐2	☐3	☐8
4. Fatigue	☐1 Neg.	☐2 Pos. → Duration?	☐1	☐2	☐3	☐8
5. Dyspepsia	☐1 Neg.	☐2 Pos. → Duration?	☐1	☐2	☐3	☐8
6. Weight change	☐1 Neg.	☐2 Pos. → Duration?	☐1	☐2	☐3	☐8
7. Bladder changes	☐1 Neg.	☐2 Pos. → Duration?	☐1	☐2	☐3	☐8
8. Bowel changes	☐1 Neg.	☐2 Pos. → Duration?	☐1	☐2	☐3	☐8
9. Other: __________	☐1 Neg.	☐2 Pos. → Duration?	☐1	☐2	☐3	☐8

Comments: ____

II. Pre-operative CA 125 screens

☐ None listed in clinic records

Date of exam	Results			
__ __ __ __ __ __ __ __	__ __ __ __ . __	U/ml	☐1 Dynacare	☐2 Other laboratory
__ __ __ __ __ __ __ __	__ __ __ __ . __	U/ml	☐1 Dynacare	☐2 Other laboratory
__ __ __ __ __ __ __ __	__ __ __ __ . __	U/ml	☐1 Dynacare	☐2 Other laboratory
__ __ __ __ __ __ __ __	__ __ __ __ . __	U/ml	☐1 Dynacare	☐2 Other laboratory

III. Size of ovarian mass

Abstract from pathology reports.

Date of report	LT	RT	Bidimensional tumor size (note units)
__ __ __ __ __ __ __ __	☐1	☐2	__ __ . __ ____ x __ __ . __ ____
__ __ __ __ __ __ __ __	☐1	☐2	__ __ . __ ____ x __ __ . __ ____

IV. Post-operative diagnosis

Record all that apply.

	Ca	LMP	Ben	Nml	
Rt. ovary	☐1	☐2	☐3	☐4	Dx.: ____________________
Lt. ovary	☐1	☐2	☐3	☐4	Dx.: ____________________

Other relevant conditions: __ __ Dx.: ____________________

__ __ Dx.: ____________________

__ __ Dx.: ____________________

ORCHID – *Surgical Reporting Form*

Patient name: ______________________ UPN: __________
LAST FIRST MI

Medical Rec. ID: __________ Procedure: __________

Surgeon ID: __________ Procedure date: ___/___/___

Institution of procedure: SMC UW

Summary of Operative Findings: 1 = Involved 2 = Not Involved 3 = Not Examined

	PELVIS	1	2	3
1	Uterus corpus	☐1	☐2	☐3
2	Vagina	☐1	☐2	☐3
3	Fallopian tube – RT	☐1	☐2	☐3
4	Fallopian tube – LT	☐1	☐2	☐3
5	Ovary – RT	☐1	☐2	☐3
6	Ovary – LT	☐1	☐2	☐3
	PERITONEUM			
7	Pelvic – RT	☐1	☐2	☐3
8	Pelvic – LT	☐1	☐2	☐3
9	Pelvic bladder	☐1	☐2	☐3
10	Pelvic cul-de-sac	☐1	☐2	☐3
11	Ant. abdomen	☐1	☐2	☐3
12	Gutters – RT	☐1	☐2	☐3
13	Gutters – LT	☐1	☐2	☐3
14	Subdiaphragm – RT	☐1	☐2	☐3
15	Subdiaphragm – LT	☐1	☐2	☐3
	BOWEL			
16	Stomach	☐1	☐2	☐3
17	Duodenum	☐1	☐2	☐3
18	Jejunum	☐1	☐2	☐3
19	Ileum	☐1	☐2	☐3
20	Appendix	☐1	☐2	☐3
21	Ascending – colon	☐1	☐2	☐3
22	Transverse – colon	☐1	☐2	☐3
23	Descending – colon	☐1	☐2	☐3

	MESENTERY	1	2	3
24	Small bowel	☐1	☐2	☐3
25	Large bowel	☐1	☐2	☐3
	ABDOMINAL ORGANS			
26	Liver surface	☐1	☐2	☐3
27	Liver parenchyma	☐1	☐2	☐3
28	Spleen	☐1	☐2	☐3
29	Gallbladder	☐1	☐2	☐3
30	Pancreas	☐1	☐2	☐3
31	Kidney – RT	☐1	☐2	☐3
32	Kidney – LT	☐1	☐2	☐3
33	Omentum – gastrocolic	☐1	☐2	☐3
34	Omentum – infracolic	☐1	☐2	☐3
	NODES			
35	Para-aortic – RT	☐1	☐2	☐3
36	Para-aortic – LT	☐1	☐2	☐3
37	Pelvic – RT	☐1	☐2	☐3
38	Pelvic – LT	☐1	☐2	☐3

OTHER FINDINGS

39 Pleural effusion ☐1 No ☐2 Yes

40 Ascites ☐1 No ☐2 Yes

41 Volume: __________ ml

42 Resection: ☐1 Suboptimal (≥ 2 cm.)

43 ☐2 Optimal, with residual:

44 ☐1 ≤ 2 cm.

☐2 ≤ 1 cm.

☐3 no residual disease

Date entered into database: ___/___/___ Data entry tech. ID: __________

Appendix E

Core Participant Follow Up Documentation

Statistical, Clinical and Laboratory Coordinating Core

Nicole Urban, ScD, Garnet Anderson, PhD, Nancy Kiviat, MD,
Leona Holmberg, MD, Jane Kuypers, PhD,
Charles Drescher, MD, Mary Anne Rossing, PhD

September 24, 1999

«firstname» «lastname»
«address»
«city», «state»

Dear Ms.,

Thank you very much for taking the time to complete and return the ORCHID study materials you received during your visit to Pacific Gynecology Specialists on «dateenrolled». While reviewing your materials, we noticed that the following items are missing or incomplete:

«Packet»

Would you be so kind as to complete and return these materials to us at your earliest convenience? Enclosed you will find a copy of <<*this form/these forms*>> for you to complete and return to us in the postage-paid envelope. In addition, I will contact you by telephone within the next two weeks to answer any questions that you may have about completing this information or about your participation in the ORCHID study.

Please feel free to contact me at 206-215-6205 should you have any questions. Thank you again for participating in the ORCHID study.

Sincerely,

Suepattra G. May
Project Coordinator

Enclosures
«upn»

Intro Hello, may I please speak to [FIRST NAME] [LAST NAME]?

Hi, Ms. [LAST NAME], this is _____________ calling from the ORCHID study. I'm calling to follow up on a letter we recently sent you about your study enrollment materials and to find out if you have received it.
[PROBE: Did you receive the letter?]

☐ Inconvenient Time [**GO TO "Inconvenient time"**]

☐ YES [Received letter and already mailed it back in – **GO TO 1**]

☐ YES [Received letter, but hasn't mailed it back in yet - **GO TO 2**]

☐ NO - go to **DID NOT RECEIVE LETTER**

1. That's great. I look forward to receiving your packet any day then. Do you have any questions for me at all at this time?

Inconvenient Time

1.a. When would be a good time to call back?

___ ___/___ ___/______@___ ___-___ ___ am/pm

Thank you. I'll try to reach you again at that time.

2. Great. Ms. [LAST NAME],
Have you had a chance to review the letter and enclosed material(s) yet?

☐ Yes (continue)
☐ No (has not yet reviewed materials) **(GO TO 4)**

3. Do you have any questions about completing the study enrollment forms?

☐ Yes (answer questions, then go to 4)
☐ No (continue)

4. In order for us to complete your enrollment for ORCHID, we will need you to (as applicable) <<<sign the consent form>>> <<< complete the questionnaire>>>, <<<complete the participant enrollment form>>> <<<complete the medical records release form>>>, and return these materials to us in the envelope we provided. Would you be able to send these materials in the next few days?

☐ Yes (continue)
☐ No (go to **DOESN'T COMMIT TO RETURN COMPLETED INFORMATION)**

Great. After we receive your <<<missing or incomplete information>>>, we will send you a letter to confirm that it's been received. Do you have any questions about this?

☐ Yes (answer questions and continue)
☐ No (continue)

Great. I look forward to receiving the completed materials from you. Have a good day.

DOES NOT COMMIT TO SEND PACKET

Ms. [LAST NAME], in order for us to complete your enrollment, we will need to receive your completed enrollment forms. Do you have an idea when you could return those forms to us?

[NOTE DATE] ______________________

Okay, Ms. [LAST NAME], I look forward to receiving your completed forms soon. Once we receive those materials we will send you a letter to confirm receipt. Do you have any questions about this? Have a good day.

DOES NOT WISH TO PARTICIPATE

Okay, I respect your decision and appreciate the time you've already given. I have just one last question. For research purposes only, we are interested in your reasons for choosing not to complete this information. Could you please tell me why you aren't interested in participating in the study?
PROBE: Are there any other reasons?

☐ Doesn't have time
☐ Demands of work
☐ Personal health
☐ Family illness, family death, emergency or other family demands
☐ Priorities other than work or family
☐ Financial problems, including unemployment
☐ Doesn't like filling out forms
☐ Doesn't like phone calls
☐ Refuses to give reason
☐ Other (specify)____
☐ Other (specify)____
☐ Other (specify)____

Thank you for taking the time to answer these questions. We appreciate your contribution to the ORCHID study.

DID NOT RECEIVE PACKET - Okay. Let me check that we have your correct address and we'll re-send the information. I show your address as [ADDRESS]. Is this correct?

YES - continue

NO - go to **DID NOT RECEIVE-A**

Great. We'll send those materials within the next day or two, and call again once you've received them. Have a good day.

DID NOT RECEIVE-A
Could you please tell me the correct address?

____

____

____

Appendix F

Core Enrollment and Histology Reports

Statistical, Clinical and Laboratory Coordinating Core

Nicole Urban, ScD, Garnet Anderson, PhD, Nancy Kiviat, MD,
Leona Holmberg, MD, Jane Kuypers, PhD,
Charles Drescher, MD, Mary Anne Rossing, PhD

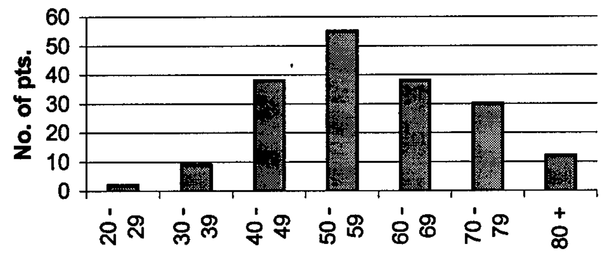
ORCHID Enrollment Report

As of: 10/28/99

Total participants enrolled:	**217**
- with enrollment & questionnaire data:	**184**

Demographics

Age Distribution (N=184)



Median = 57

Ethnicity	White	88.0%	(161/183) 1M
	Asian, Pac. Islander	5.5%	(10/183) 1M
	Hispanic/Latino	0.5%	(1/183) 1M
	Black/African Amer.	1.1%	(2/183) 1M
	Native Amer., Amer. Indian	3.3%	(6/183) 1M
	Other	1.6%	(3/183) 1M
Education	HS education or less	24.0%	(44/183) 1M
	Greater than HS education	76.0%	(139/183) 1M
State of residence	Washington State	92.2%	(200/217)
	Other	7.4%	(16/217)

Medical History

Median age at menarche:	13		(N=179) 5M
Menopausal status	Still having periods	19.9%	(33/166) 18M
	Possibly going through menopause	7.2%	(12/166) 18M
	Periods stopped by themselves	32.5%	(54/166) 18M
	Periods stopped through surgery	28.3%	(47/166) 18M
	Still having periods due to HRT	6.0%	(10/166) 18M
	Other	6.0%	(10/166) 18M

Cancer History

Self-reported breast cancer diagnosis:	9.8%	(18/183) 1DK
Self-reported ovarian cancer diagnosis:	25.7%	(44/171) 2M 11DK
Self-reported colon cancer diagnosis:	1.1%	(2/184)
Self-reported diagnosis of other ca.:	19.1%	(34/178) 2M 4DK
Identified at least one relative with ovar. ca.:	8.8%	(16/181) 3DK
- of these, pts. identifying a 1st deg. relative:	50.0%	(8/16)
Identified at least one relative with breast ca.:	38.3%	(70/183) 1DK
- of these, pts. identifying a 1st deg. relative:	60.0%	(42/70)
Identified at least one relative with colon ca.:	19.9%	(34/171) 2M 11DK

DK = don't know; M = missing

10/28/99

ORCHID Specimen Histology

As of: 10/28/99

Enrollments with specimen collection: 181

Enrollments with serum collection only: 17

Enrollments with ovarian tissue collection: 146

			Stage				
			I	*II*	*III*	*IV*	*unknown*
Cancer:		**39**					
	Serous	21	*0*	*0*	*17*	*4*	*0*
	Mucinous	3	*3*	*0*	*0*	*0*	*0*
	Endometrioid	4	*3*	*0*	*1*	*0*	*0*
	Other (a)	11	*1*	*2*	*5*	*3*	*0*
LMP:		**6**					
	Serous	4	*3*	*1*	*0*	*0*	*0*
	Mucinous	2	*2*	*0*	*0*	*0*	*0*
	Endometrioid	0	*0*	*0*	*0*	*0*	*0*
	Other	0	*0*	*0*	*0*	*0*	*0*
Benign:		**31**		*not applicable*			
	Serous	6					
	Mucinous	3					
	Endometrioid	0					
	Brenner	1					
	Non-neoplastic (b)	9					
	Other (c)	12					
Normal:		**57**		*not applicable*			
Awaiting pathology diagnoses:		**13**					
Other collections: (non-ovarian primary/no ovaries collected)		17					

(a) Other cancers:

(b) Non-neoplastic other:

(c) Other benigns:

Appendix G

Publications

Project 1

Identification of Potential Markers for Population Based Screening for Ovarian Cancer: Characterization of Differential Gene Expression in Malignant Neoplasia by Use of High Density Array Hybridization (HDAH).

ELSEVIER

Gene 238 (1999) 375–385

GENE
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Comparative hybridization of an array of 21 500 ovarian cDNAs for the discovery of genes overexpressed in ovarian carcinomas

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Abstract

Comparative hybridization of cDNA arrays is a powerful tool for the measurement of differences in gene expression between two or more tissues. We optimized this technique and employed it to discover genes with potential for the diagnosis of ovarian cancer. This cancer is rarely identified in time for a good prognosis after diagnosis. An array of 21 500 unknown ovarian cDNAs was hybridized with labeled first-strand cDNA from 10 ovarian tumors and six normal tissues. One hundred and thirty-four clones are overexpressed in at least five of the 10 tumors. These cDNAs were sequenced and compared to public sequence databases. One of these, the gene *HE4*, was found to be expressed primarily in some ovarian cancers, and is thus a potential marker of ovarian carcinoma. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Cancer maker; DNA array; Differential expression; HE4

1. Introduction

Ovarian cancer is the leading cause of gynecological cancer death in the United States. The American Cancer Society estimates that in 1998, some 25 400 women will develop ovarian cancer and 14 500 will die from it (American Cancer Society, 1998). The overall 5 year survival rate is about 46%, and has remained essentially unchanged for 25 years. Ovarian cancer is ranked fifth in cancer mortality among women, and raises concerns both with women and physicians because of its generally poor prognosis. Cancers diagnosed at an early stage have a 5 year survival rate of 92% in contrast to a 25% 5 year survival rate for patients with disseminated disease at diagnosis. Seventy-five per cent of epithelial ovarian cancers are diagnosed at advanced stages. This is in part due to the lack of symptoms early in the disease course, and the absence of a sensitive and specific screening test for early disease detection. Currently available ovarian cancer markers such as CA-125 are neither sensitive nor specific enough for population screening to detect early, treatable ovarian cancers (Jacobs et al., 1993).

We describe the use of 'high-density cDNA array hybridization' (HDAH) to identify transcripts that show high expression levels in ovarian cancer tissues as compared to ovarian surface epithelium (OSE). This technology has been used in a variety of experiments to identify transcripts (Schena et al., 1998), whose expression patterns differ in two tissues (e.g. normal and cancer). Our objective is to find (1) transcripts that are overexpressed in tumor as contrasted with normal ovarian tissue and (2) cDNAs encoding proteins that could be useful diagnostic markers (e.g. secreted or cell-surface pro-

Abbreviations: bp, base pair(s); cDNA, copy DNA; EST, expressed sequence tag; HDAH, high-density array hybridization; HE4, human epididymis gene 4; kb, kilobase(s); nt, nucleotide(s); OSE, ovarian surface epithelium; PBL, peripheral blood lymphocytes; RT-PCR, reverse transcription polymerase chain reaction.

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0378-1119/99/$ – see front matter
PII: S0378-1119(99)00342-X

teins). Two general types of assays are possible: (1) protein assays for secreted proteins or on the surface of cells that metastasize into the circulation, and (2) PCR assays from genes uniquely expressed in blood-borne (or ascites-borne) tumor cells. Hybridizing 21 500 randomly selected cDNAs from normal and neoplastic ovarian tissues with probes from 10 ovarian tumor and six normal tissues, we identified 134 clones with higher expression signals in ovarian tumors as opposed to normal tissues. These clones were sequenced, and in some cases, their expression pattern was confirmed by RT-PCR and Northern blot analysis. The expression pattern of one of these clones, *HE4*, suggests that it may be a potential candidate diagnostic marker for ovarian cancer.

2. Materials and methods

2.1. Tissues and cells

We used the following tissues for our experiments: ovarian surface epithelium short-term culture (Karlan et al., 1995), early passages (OSE); normal ovary consisting of primarily stromal cells (N002, N005, N006, N019 and N035); two benign ovarian tumors (T017B, an endometrioid polyp, and T018B, a serous cystadenoma); one borderline early stage serous carcinoma, LMP (T028L); late-stage, high-grade papillary serous ovarian adenocarcinomas (T001–T006, T008–T011, T014–T016 and T021); two early-stage ovarian adenocarcinomas (one serous: T007 and one mucinous: T037); one late-stage, high-grade serous ovarian adenocarcinoma post-chemotherapy (T012); two late-stage, high-grade serous ovarian adenocarcinoma with massive metastases (T013M and T026M); peripheral blood lymphocytes (PBL1 and PBL2); Fetal ovaries: pool of 25 fetal ovaries (52–103 days); bone marrow, cerebellum, kidney, liver and placenta (Clontech, Palo Alto, CA). In order to minimize the effect of variance in tissue collection on the RNA quality and hence the hybridization patterns, we ensured that tissue collection would adhere to the following guidelines. After surgery, a tissue section was taken for the pathologist's examination and an adjacent section was snap-frozen in liquid nitrogen. All ovarian tumor tissue specimens were examined for their tumor cell content (which was above 80%) and the absence of necrosis. RNA preparations of all tissues or cell cultures were performed using the Trizol method (Life Technologies, Grand Island, NY). Poly$(A)^+$ RNA was prepared using a mRNA purification kit (Stratagene, La Jolla, CA). Tissue samples of 200–400 mg of tumor were used for RNA preparation. We have found that samples of less than 200 mg do not yield sufficient RNA for our analysis. The integrity of total RNA was determined by visual inspection of the 28S and 18S ribosomal bands to ensure that degraded samples that might give a different expression profile than intact RNA were not used.

2.2. Minipreparation of 21 500 ovarian clones

Five cDNA libraries were created from ovarian tissues and cell cultures (OSE, T007, T008, T010 and T012) using the ZAP-cDNA synthesis kit (Stratagene). Examining the cDNA clones using PCR, the insert sizes were found to average between 1.2 and 1.5 kb. From each library, 96 clones were randomly chosen, sequenced and analyzed by similarity analysis against the non-redundant and EST database. The low number of mitochondrial and ribosomal sequences, the limited number of clones with no insert, and the significant cDNA diversity indicated that the libraries were of high quality. Using a 96-deep-well plate-based minipreparation assay (Ng et al., 1996), we picked 21 500 transformants (8600 from the OSE cDNA library and 3225 each from the four tumor cDNA libraries), extracted the cDNAs and transferred them to 384-well microtiter plates.

2.3. Dotting the 21 500 clones onto nylon membranes

Using a hand-held arraying tool with a 384-pin printhead developed in our laboratory (Schummer et al., 1997), we dotted the 21 500 cDNAs onto 16 sets of 14 nylon membranes of 7.5×12 cm, which held each of the 1536 clones. The cDNA was denatured and immobilized on the membrane as previously described (Schummer et al., 1997).

2.4. Labeling and hybridization protocol

Each set of membranes was hybridized with a complex probe consisting of ^{32}P-labeled first-strand cDNA. Briefly, 5 μg of poly(A^+) RNA or 30 μg of total RNA were reverse-transcribed using Superscript II reverse transcriptase (Life Technologies) and oligo-dT_{12} primers with 30 μCi of alpha-^{32}P-dCTP (3000 Ci/mmol) and unlabeled dATP, dGTP, dTTP at 1 mM each; after 20 min, unlabeled dCTP was added to a final concentration of 1 mM, and the reaction was continued for another 40 min. This unpurified probe was hybridized to 12 membranes under conditions described previously (Schummer et al., 1997). The membranes were washed at increasing stringency (20 min, $2\times$ SSC, 0.5% SDS, RT; 20 min $0.5\times$ SSC, 0.5% SDS, 65°C; 2×20 min, $0.2\times$ SSC, 0.5% SDS, 65°C).

2.5. Software for spot detection

After hybridization and washing, the membranes were exposed to a phosphor storage screen, and the hybridization patterns were captured as 16-bit TIFF

images using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Nine nylon membranes were imaged simultaneously on a 35 × 45 cm screen. The resulting file was processed using a software package developed in our laboratory. The TIFF image was split into nine smaller images, each representing one of the arrayed membranes. Briefly, the user defined the outer dimensions of each membrane by placing a cursor into each the upper left, upper right and lower right corner of each of the nine array images. Subsequently, the computer superimposed a grid, approximating the positions of the 1536 dots. By five passes of center-of-mass finding, the computer determined the exact center of each of the 1536 dots. It integrated the area of an experimentally determined number of pixels around each center that covered the area of the largest hybridization signal present on the membranes. The intensities of all pixels in the area were integrated. Local background was calculated by choosing one pixel with the lowest intensity out of four pixels situated halfway between one dot and its four diagonal neighbors. Both values were stored in a tab-delimited text file together with the coordinates of the spot on the array.

2.6. Single pass 5′ sequencing, database analysis and sequence comparison

Sequencing was performed on plasmid DNA and PCR products using previously described methods (Ng et al., 1996). The single-pass sequences were edited to remove vector and poly(A) sequences. Edited sequences were compared with those in the EST (dbEST) and non-redundant nucleotide and protein databases (GenBank) at the National Center for Biotechnology Information (NCBI) using the Baylor College of Medicine Search Launcher batch client server 'Search Launcher' (http://www.hgsc.bcm.tmc.edu/Search Launcher/). Nucleotide sequence comparisons were carried out using BLASTN. Comparisons of conceptual protein translations were performed using the program BLASTX with BEAUTY sequence annotation enhancement. Each clone was categorized as to known gene homology, EST homology, or novel.

2.7. RT-PCR

Clones determined by to be differentially expressed by array analysis were confirmed by single tube RT-PCR, which has been shown to be a highly sensitive measure of transcript abundance (Schummer et al., 1998). Two primers, with a base pair length of 20–24 and with T_ms between 64 and 66°C, were designed for each gene. The distance between the primers was 420–660 bp. RT-PCR (Titan®, Boehringer Mannheim, Mannheim, Germany) was performed with 200 ng of total RNA according to the manufacturer, with the following cycles: 30 min at 50°; 2 min at 94°; 10 cycles of 30 s at 94°, 30 s at 60°, 45 s at 68°; 12–25 cycles of 30 s at 94°, 30 s at 60°, 45 s at 68° (with elongation of 5 s for each cycle); 7 min at 68°. For each gene, the logarithmic phase of amplification was determined prior to the Titan®-PCR. The individual reactions were run on a 1% agarose gel stained with SYBR-Green at 500 × diluted concentration for 1 h and scanned on a FluorImager (Molecular Dynamics, Sunnyvale, CA). For each gene and tissue, four identical reactions were performed.

2.8. Northern blot

A *HE4* PCR product of 500 bp was cloned into a pCR2.1 vector using the TA cloning kit (Invitrogen, San Diego, CA). A digoxigenin-labeled riboprobe was prepared from this vector using a Genius RNA DIG labeling kit (Boehringer Mannheim, Germany). The probe was hybridized overnight at 68°C in DIG Easy Hyb buffer and washed in 2 × SSC, 0.1% SDS for 15 min at room temperature; 2 × SSC, 0.1% SDS for 20 min at 68°C; and 0.1 × SSC, 0.1% SDS for 2 × 15 min at 68°C. The hybridized RNA was visualized using the DIG detection kit (Boehringer Mannheim), and the membrane was exposed to X-ray film for 15 min.

3. Results and discussion

3.1. Evaluation of high-density filter hybridization

Tissues comprise many different cell populations. Each type of cell in a tissue exhibits its particular gene expression pattern. Since most ovarian tumors arise from epithelial cells, the comparison of tumors against ovarian surface epithelium should provide a useful comparison. Two qualifications must be made: (1) ovarian surface epithelial cells in a short-term culture will probably have some differences in expression patterns from in-vivo ovarian epithelial cells, and (2) tumors may have intermixed normal cells from the ovary. In order to detect genes that are overexpressed in one cell type or tissue versus another, one needs to know the limitations of the detection system, notably (1) the upper and lower limits of detection (signal-to-noise ratio) which — translated into the number of mRNA molecules detectable per cell — should be suitable for the proposed study, and (2) the measured level of variation in signal intensity on identical membranes interrogated with identical probes. The latter will determine a factor above which overexpression can be regarded as significant.

3.1.1. Determination of detection limit and dynamic range

The sensitivity of the array technology determines the number of detectable mRNA molecules in a cell. In order to determine the mean signal-to-noise ratio, we hybridized 14 identical arrays containing 1536 identical cDNAs coding for the green fluorescent protein (*GFP*) with first-strand cDNA probes made from human liver poly$(A)^+$ RNA in which a *GFP* mRNA was added in decreasing concentrations (14 different concentrations ranging from one transcript in 200 to one in 20 000). As depicted in Fig. 1, the probe with the highest *GFP* concentration yielded a mean value of 8300 ± 416 dpm (decays per minute) per pixel, and the mean background value was determined as 90 ± 18 dpm/pixel. With background subtraction, this represents a dynamic range of 456 (background-subtracted signal divided by background fluctuation: $8210/18 = 456$) or 2.5 orders of magnitude. We established a lower limit of sensitivity of 1 *GFP* RNA in 20 000 liver RNAs, a result similar to those in other studies (Piétu et al., 1996). Based on an estimated 10^5–10^6 transcripts per average eukaryotic cell (Bishop et al., 1974), the membrane-based HDAH can detect a minimum of between five and 50 mRNA molecules in a cell and a maximum of 500–5000. The lower limit falls in the low to medium class of transcripts, and the upper limit lies in the highly expressed gene class (Zhang et al., 1997). This detection range should be sufficient for the identification of overexpressed genes.

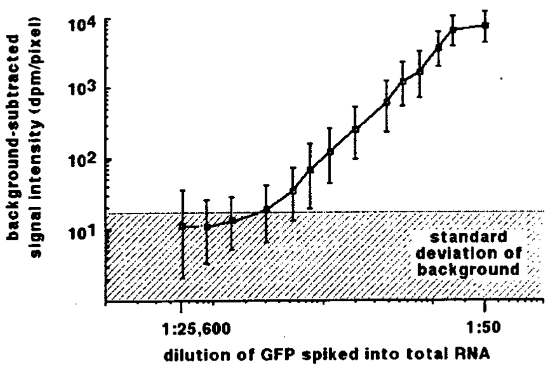

Fig. 1. Determination of the linearity of the hybridization signal. Fourteen replica membranes with 1536 *GFP* cDNAs each were hybridized with first-strand cDNA made from poly$(A)^+$ RNA from human liver with *GFP* mRNA spiked into it in a twofold serial dilution starting with a 1:50 dilution. The signal intensity is measured as the average of all pixels of all 1536 signals. Displayed are the background-subtracted intensities with their standard deviations. The resulting curve is linear over 2.5 orders of magnitude). The standard deviations increase with decreasing signal-to-noise level. The shaded area indicates the standard deviation of the background. The background intensity averaged at 90 ± 18 dpm/pixel, and the highest intensity averaged at 8300 ± 416 dpm/pixel.

3.1.2. Normalization of the hybridization signals

In order to compare hybridization signatures of two identical membranes that have been hybridized with different probes in two separate incubations, one needs to normalize the signals to a standard. Although we adhere to a strict protocol, slight variations can be introduced by minute differences in probe labeling, probe purification, hybridization and wash conditions and exposure time. We normalized the background-subtracted intensities of one membrane by setting the median to 1. Assuming that among the 1536 clones present on one membrane, the majority does not alter its expression (Zhang et al., 1997), we believe that this is justified.

3.1.3. Determination of variation in signal intensity

Two factors influence the accuracy of the hybridization detection for one particular cDNA on a membrane: the amount of cDNA on the membrane (governed by the dotting procedure) and the amount of labeled cDNA that remains bound to the target cDNA on the membrane after hybridization (governed by the efficiency of the probe labeling reaction and the hybridization and washing kinetics). We determined the variation of amounts of DNA spotted by our arraying tool to be $\pm 14\%$ (data not shown). Since the probe consists of a complex mixture of cDNAs, the arrayed DNA is in vast excess of the probe cDNA, and thus the variations caused by the spotted cDNA can be regarded as negligible. In order to assess the probe-to-probe variance, we hybridized four replica membranes containing 1536 ovarian cDNAs with four ^{32}P-labeled first-strand cDNA probes independently generated from one batch of total RNA prepared from liver tissue. We compared the background-subtracted intensities of one cDNA across the four membranes and calculated the standard deviation, thus generating 1536 values. We ranked the clones by their expression and determined three means of standard deviations, one for the upper, the middle and the lower third, corresponding roughly to the high, medium high and low, expression categories of transcripts. The mean of the standard deviations amounted to $\pm 15\%$, $\pm 24\%$ and $\pm 40\%$ respectively, which averages to $\pm 26\%$ for all clones. Using the following equation, we calculated the threshold value for a ratio to be regarded as significant: $[1 + \text{standard deviation}]/[1 - \text{standard deviation}]$. In order to be above this threshold of significance, a highly expressed gene needs to display a ratio of 1.35, a medium expressed gene a value of 1.63 and the least expressed gene a value of 2.33. These measurements would suggest that a threshold of significance, which is a function of intensity, should be used and that the threshold will vary from 1.35 for the most highly expressed genes to 2.33 for the least expressed genes. However, the measurements performed here are at best a surrogate system for estimating

error in the tumor data, i.e. the above experiments control for hybridization, filter and analysis variation but do not control for labeling and other sample-handling variation in the tumor samples. With limited tissue available for each tumor, it is not possible to perform replicate measurements on all our samples to generate similar significance curves for the actual data. Hence, we chose to use a ratio of 2.5 or more as the threshold of significance for our tumor data. We recognize that this criterion will result in the exclusion of genes that are differentially regulated at a statistically level. However, given that ourgoal is to develop genes that may serve as serum markers for ovarian cancer, and given the limitations of currently available assay systems for serum marker testing, a factor of 2.5 differential expression is appropriate.

3.2. Screening of 21 500 ovarian clones

An ideal array of cDNAs would contain a single copy of every gene expressed by the tissues to be compared. Since the identification of all human genes is incomplete, we chose to array randomly selected cDNAs derived from a wide spectrum of ovarian tissues including normal ovarian epithelium, early stage ovarian carcinomas, and late-stage pathologically aggressive ovarian carcinomas. We chose to array 8600 clones in form of purified plasmids from an OSE library [short-term culture of ovarian surface epithelial cells (Karlan et al., 1995)], and 3225 each from four ovarian cancer cDNA libraries from increasing malignancy, totaling 21 500 arrayed clones. We created 16 replicate sets of these arrays, each set consisting of 14 membranes of 7 × 12 cm holding 1536 clones. Each of the membrane sets was hybridized with a ^{32}P-labeled first-strand cDNA probe made from the RNA of an early-stage serous ovarian tumor (T007), eight late-stage serous ovarian tumors (T004, T008, T009, T010, T011, T014, T015, T016), one recurrent ovarian tumor (T012), ovarian surface epithelium (N001S), liver, placenta, bone marrow, cerebellum, and kidney. Two types of comparative experiments were carried out: (1) normal and tumor ovarian tissues were contrasted, and (2) ovarian tissues were compared against a variety of normal tissues. The first comparisons would reveal the tumor-specific cDNAs and the second the ovarian-specific cDNAs (at least with respect to the five different normal tissues). It was not our purpose to analyze early-to-late stage differences or tumor stratification as the limited number of cancerous tissues would not allow this. Our objective was to determine whether it is possible to use this technique to detect genes that are overexpressed in ovarian carcinomas respective to normal ovary and other tissues.

3.3. Differential transcript expression

Using the spot-finding and detection software developed in our laboratory, we determined the hybridization intensities for each clone and calculated their ratios. Comparing the 10 hybridizations with ovarian tumor tissues to those with OSE, the vast majority (>93%) of the clones displayed tumor-to-OSE ratios of less than a factor 2.5, and therefore were considered unchanged; about 7% of the clones exhibited a tumor-to-OSE ratio of more than 2.5, 0.9% a ratio of greater than 5.0, and 0.5% a ratio of greater than 10.0. Thus, most transcripts were expressed at similar levels in normal and tumor tissues, a finding that has been reported in colorectal and pancreatic cancers (Zhang et al., 1997).

No clone exhibited a 2.5-fold difference in expression in more than six of the ovarian tumors relative to OSE. Given the difference in tumor stages (one was an early stage tumor, and one a recurrent late stage tumor, the rest being late-stage ovarian adenocarcinomas) and the fact that the same stages, if they represent different stratified types, do not necessarily reflect high degrees of similarity on the molecular level; given the inter- and intra-tissue heterogeneity (possible proximity of section to areas of necrosis, differences in histology and pathology between tumors and across tumor sample), we did not expect to see a particular clone exhibit high tumor-to-OSE ratios in all tumors.

Sixteen clones showed overexpression in at least six ovarian cancers, but 14 of these 16 were also expressed in at least one non-ovarian tissue. In order to obtain a reasonable number of clones with overexpression in ovarian tumors and not in non-ovary tissues, we chose clones that fulfilled the following criteria: ratios greater than 2.5 in at least five out of the 10 tumors compared to OSE, and ratios below 2.5 in bone marrow, cerebellum, kidney, liver, and placenta compared to OSE. We were able to identify 134 clones that fulfilled these criteria. Sequencing of the partial cDNA clones revealed 60 that matched sequences in the non-redundant (nr) GenBank database. Of these, 17 matched to mitochondrial and ribosomal genes, and 43 matched to 37 other characterized genes (Table 1). Forty-seven clones matched only to sequences in the EST database, and 24 clones did not match any sequence in GenBank and were classified as novel. Three clones of 254, 312 and 323 bp length matched entirely to SINE and LINE sequences and were thus classified as repeats (see Table 1).

The expression patterns of two of these clones, which code for *S-adenosyl homocysteine* hydrolase and *HE4*, are shown in Fig. 2. For both genes, the calculated overexpression by signal intensities in the cancer tissues can be confirmed by visual inspection of the hybridized membranes. It is obvious, however, that by visual inspection alone, these clones would have probably escaped our scrutiny since their expression is rather weak compared to neighboring clones.

The overexpression of the 17 clones with similarity to mitochondrial sequences and ribosomal proteins can

Table 1
Categories of cDNAs present in the 134 clones[a]

Number of sequences	Percentage	Sequence similarity
3	2	Repeats
6	4	Mitochondrial sequences
2	2	Ribosomal RNA
9	7	Ribosomal proteins
24	18	Novel sequences
47	35	ESTs (expressed sequence tags)
43	32	Known genes
134		Total

[a] Novel sequences had less than 60% similarity to either human or non-human sequences. Repeats: genomic, SINE (ALU, MIR) and LINE (LINE1 and LINE2), LTR elements (MaLRs, Retroviral, MER4 group), DNA elements (MER1, MER2, Mariners). GenBank Accession Nos of the clones with similarity to known genes: *14.3.3*, X56468 (2×); *Actin capping protein*, U03269; *alpha-enolase*, M14328; *beta-actin*, M10277; *beta-2 microglobulin*, M17987; *BA46*, U58516; *Catechol-O-methyltransferase*, M65212; *CD44*, L05412; *CLIP/Restin*, M97501/X64838; *E16*, M80244; *Elongation factor 1 beta*, X60489; *Elongation factor 1 gamma*, Z11531 (2×); *Elongation factor 2*, Z11692; *Flightless*, U01184; *HE4*, X63187 (2×); *Initiation factor 4AI*, D13748; *Insulin-like growth factor BP 3 precursor*, M31159; *MDC15*, U46005; *Mucin*, X52229; *Myosin*, M22918; *Oviductal glycoprotein*, U09550 (3×); *p84*, L36529; *Peroxisomal targeting signal receptor I*, U19721; *Phosphatidyl inositol-3-kinase* alpha subunit, M61906; *Poly-A binding protein*, Y00345; *Procollagen alpha COL1A2*, K01078; *putative Progesterone binding protein*, Y12711; *Proteasome subunit HC8*, D00762; *RhoA*, L25080; Ryudocan, D13292; *S-adenosyl-homocysteine hydrolase*, M61831; *Smooth muscle protein*, M95787; *Tenascin precursor*, X56160; *Thiol-specific antioxidant*, Z22548; *Thymosine beta 4* (interferon-inducible), M17733; *Tropomyosin*, M75165; *Ubiquitin*, M10939, X56997 (2×).

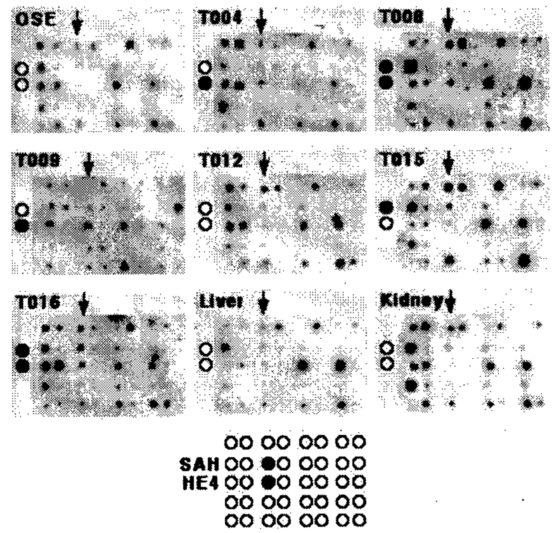

Fig. 2. Visual inspection of the membranes after identification of differentially expressed clones through computing the signal intensities. Displayed are eight columns and five rows of close-ups on nine membranes that have been hybridized with nine different probes. Two clones, coding for *HE4* and *S-adenosyl homocysteine hydrolase* (*SAH*), show high tumor-to-OSE ratios and low normal-to-OSE ratios (see Table 2). The positions of these two clones on the array are marked in the bottom panel. The clone positions on the nine membranes are indicated by an arrow on top of each panel and the circles on the left. An empty circle denotes a weak hybridization signal, and a filled circle denotes a strong signal.

be attributed to the higher metabolic activity of the tumors. Ribosomal protein sequences have been found to be more highly expressed in colon carcinomas (Pogue-Geile et al., 1991). Likewise, five other genes linked to metabolic pathways such as *elongation factor 1 gamma* and *initiation factor 4AI* were overexpressed in ovarian cancer tissues. It is notable that these 22 clones displayed an average tumor-to-OSE ratio of 5.22 ± 2.4, whereas the remaining 38 clones with homology to known genes had a lower average ratio of 4.11 ± 1.8. This underscores the fact that the degree of overexpression alone is not necessarily indicative of a clone that can be used as a marker protein.

In order to estimate the quality of the HDAH in identifying cancer related genes, and since we were realistically capable of processing only a limited number of clones, we focused on the 43 previously characterized clones, as opposed to the 47 clones that match only ESTs or those 24 that do not match any sequence in GenBank. Of the 43 clones with homology to the 37 characterized genes, 10 genes are expressed in epithelial tissues: *14.3.3*, *BA46*, *CD44*, *HE4*, *Mucin1*, *Oviductal glycoprotein*, *Collagen COL1A2*, *Putative progesterone binding protein*, *RhoA*, and *Ryudocan*(GenBank Accession Nos listed in Table 1). This coincides with the fact that the vast majority of ovarian cancers, including all those used for HDAH, arise from the ovarian surface epithelium (Berchuck et al., 1996).

Thirteen of the 37 genes (35%) are known to be overexpressed in various cancers, including lung, breast and colon. Six of these 13 are expressed in ovarian carcinomas, their expression not being restricted to ovarian tissues. The thirteen genes are *14.3.3* [lung cancer (Nakanishi et al., 1997)], *beta-actin* [AML (Blomberg et al., 1987) and colorectal carcinomas (Naylor et al., 1992)], *BA46* [breast cancer (Couto et al., 1996)], *CD44* [ovarian cancer cell lines (Stickeler et al., 1997)], *Clip/Restin* [Hodgkin disease and anaplastic large-cell lymphoma (Delabie et al., 1992)], *Collagen COL1A2* [ovarian cystadenoma (Kauppila et al., 1996)], *E16* [colorectal carcinoma, adenocarcinomas from breast and endometrium (Wolf et al., 1996)], *Insulin-like growth factor BP 3* [breast cancer (Ng et al., 1998)], *Mucin1* [epithelial ovarian cancer (Dong et al., 1997)], *Procollagen-alpha* [ovarian cystadenocarcinoma (Kauppila et al., 1996)], *putative Progesterone binding protein* [ovarian cancer (Isola et al., 1990)], *RhoA proto oncogene* [*ras* activation (Khosravi-Far et al., 1995)], and *MDC15*, a metalloprotease [some metalloproteases are elevated in ovarian tumor cell cultures (Fishman et al., 1997)]. These findings indicate that our approach

is indeed capable of narrowing down the pool of 21 500 randomly selected clones to a few epithelium- and cancer-related genes.

3.4. Confirmation of overexpression of four selected clones by RT-PCR-based transcript quantitation

Any clone with its expression restricted to ovarian carcinomas can be potentially used as a marker without knowing its function. Early detection of ovarian cancer, however, requires that the assay be suitable for routine screening of women, which means that it must be affordable, non-invasive and with a high degree of specificity. Only a serum-based assay can deliver this. Therefore, knowing whether a protein is secreted or membrane-bound maximizes the chance that the protein or its degradation product will be found in the blood either as freely circulating protein or bound to the membrane of a cell that has detached from the tumor. In both cases, an antibody can be used to detect the protein in the blood. A circulating cancer cell can be detected by an RT-PCR assay or fluorescence-activated cell sorting.

In an attempt to find out whether one of the 43 clones that match characterized genes would be a potential candidate for a marker protein in a serum-based assay, we examined which of the clones codes for a cell surface protein such as Her2/neu, used as a target in breast cancer treatment (Baselga et al., 1998) or a secreted protein such as Prostate Specific Antigen (PSA) which is used in prostate cancer diagnosis (Rittenhouse et al., 1998).

From the 43 clones with homology to the 37 known genes, we chose five that are expressed at the cell surface (*progesterone binding protein*, *ryudocan*, *mucin1*, *E16*, *BA46*) and one which is secreted (*HE4*). In addition, we included the gene *14.3.3*, which is expressed in the cytoplasm but which, like *HE4*, appeared twice in our selected clones list. *Beta actin* is often used as a control for quantitative analyses because of its assumed uniformity in expression in a large array of tissues. Our HDAH results suggest, however, that *beta actin* is differentially expressed in some ovarian tumors. We therefore chose to verify *beta actin* expression as well. The characteristics of the eight chosen genes are summarized in Table 2. We used RT-PCR-based transcript quantitation to confirm overexpression in tumors relative to normal tissues.

Due to the small size of our tumor specimens (ranging from 200 to 400 mg per tissue), the RNA preparations used in the array hybridization were exhausted during library construction and probe preparation. Therefore, new ovarian adenocarcinomas matching the stage and grade of the original tumors were used for the RT-PCR analysis. We chose one early-stage, low-grade mucinous ovarian adenocarcinoma (T037) five late-stage, high-grade serous ovarian adenocarcinomas (T001–T006 and T021) and two metastatic ovarian serous adenocarcinomas (T013M and T026M). In order to incorporate different tumor histologies, we included two benign ovarian tissues (T017B and T018B) as well as a borderline ovarian tumor tissue (T028L). In addition, we tested the expression in four normal ovaries (N002, N005, N006 and N019), in a pool of fetal ovaries and in two batches of peripheral blood lymphocytes (PBL1 and PBL2). The reason for analyzing the expression patterns of these genes in peripheral blood lymphocytes is to determine whether they are expressed in blood elements, for if they are, they would not be good candidates for a diagnostic probe in blood samples. The OSE, as well as the liver and placental tissue were the same as used for array hybridization. As a control for the quality of the RNA template, we included a gene that we found to be expressed at high levels in all tissues tested so far, *S31iii125* (GenBank Accession No. U61734, Trower et al., 1996).

Fig. 3 shows the results of the RT-PCR. The quantitated intensities of the PCR bands are summarized in Table 2. While trying to match the tumor tissues in stage and grade, we did not expect an exact reproduction of the ratios from the HDAH analysis. In spite of these shortcomings, we were able to reproduce the tumor-to-OSE ratios observed in the HDAH for seven out of the eight genes, albeit only qualitatively. For the gene *14.3.3*, the tumor-to-OSE ratios were low but still measurable. This discrepancy can be attributed to the difference in tumor samples used or to an erroneous reading of the HDAH signals. For three genes (*BA46*, *E16* and *Ryudocan*), a high placenta-to-OSE ratio stands in discordance with the HDAH results where they had been low. Since the placental RNA used in both cases was the same, and since our quadruple RT-PCR approach is more accurate than the HDAH method, we must conclude that in the HDAH, the placental values must have been misread for these three clones.

14.3.3 shows no tumor-to-OSE ratios above the threshold of significance of 2.5. It displays a mean ratio of 1.5 in four invasive and in one benign ovarian tumor, which does not compare well with the mean ratio of 4.4 determined in the HDAH.

BA46 shows tumor-to-OSE ratios above 2.5 in five tumors but also in one normal ovary and in placenta. In spite of its low expression in PBL (which, as noted in the beginning of this section, is a prerequisite for a serum marker), the relatively low mean ratios in RT-PCR and HDAH of 3.2 make it a second choice marker gene.

Beta actin shows tumor-to-OSE ratios above 2.5 in 10 out of the 12 tumors (a mean of 3.9 compared to 4.4 in the HDAH), but also in some normal tissues, including PBL. Although these numbers do not warrant the consideration as a tumor marker gene, they give cause to question the use of *beta actin* as a normalization standard.

Table 2
HDAH (top) and RT-PCR ratios (bottom) of nine selected genes[a]

Gene name Accession No. Protein	14.3.3 X56468 Cytopl.	14.3.3 X56468 Cytopl.	BA46 U58516 Membr	β-actin X00351 Cytopl.	E16 M80244 Membr	HE4 X63187 Secreted	HE4 X63187 Secreted	Mucin1 X52229 Membr.	ProgBP Y12711 Membr	Ryu D13292 Membr
T004				3.1		5.1	3.6		8.9	3.1
T007 early	5.9	3.7			5.5	3.0	2.7	2.6		
T008			4.1			5.1	4.9	2.6	9.9	5.0
T009	2.7			8.5		5.1	5.5		2.8	
T010	5.5	4.3	2.7	2.5						
T011			2.6		3.0		2.7			
T012 recur						2.8		8.0	3.6	
T014	6.8	3.0		4.2	5.2			5.9	7.3	
T015	6.0	2.7	2.7		4.5				2.9	4.1
T016		3.1	4.0	3.6	3.7	2.5	2.6	8.4		4.6
Liver				3.8						2.5
Placenta			2.5	3.4	9.8				1.3	2.1
PBL1	1.3	1.3		3.9					5.8	2.1
PBL2				4.7	3.9			2.0	4.1	2.3
Fetal	2.8	2.8	1.9	1.3	8.9	7.9	7.9	9.7	4.9	1.9
N002			2.1	3.8	2.4			3.7	1.8	1.4
N005			1.3	3.4	6.5	1.2	1.2	2.4	2.1	1.0
N006			2.6	3.5	4.5	2.0	2.0	2.3	4.1	0.6
N019									3.2	0.3
N035			3.1	4.2				2.1		1.3
T017B	1.6	1.6	1.6	2.7	2.0	6.6	6.6	2.3	2.6	5.3
T018B			2.5	3.6	3.3	8.8	8.8	2.8	7.8	
T028L				2.9	1.1	8.2	8.2	3.2	1.3	4.9
T037 early			1.9	3.2	1.4	1.6	1.6	7.9	3.1	4.4
T002			1.0	3.0	1.6	12.0	12.0	2.4	2.7	3.3
T003	1.6	1.6	3.7	3.1	1.5	16.0	16.0	1.9	4.7	3.7
T005			3.0	3.6	9.4	17.0	17.0	2.4	2.0	4.8
T006				2.5		9.7	9.7		2.8	2.1
T001	1.3	1.3	1.1	2.4	2.2	11.4	11.4	2.4		2.7
T021			3.0	5.7	1.2	12.3	12.3	2.5	2.1	3.7
T013M	1.9	1.9		5.2	3.4	14.1	14.1	7.3	9.7	1.4
T026M	1.2	1.2	4.0	5.8	1.9	2.8	2.8	4.5	3.5	

[a] Eight genes out of the 43 clones that match to 37 known genes were validated for their expression by RT-PCR (see Fig. 3). The volumes of the PCR bands were calculated using the software QuanitityOne (BioRad, Hercules, CA). Titan RT-PCR amplifies the template semiquantitatively; therefore, the numbers in this table are merely indicative of a tendency and cannot be translated into copy numbers. The rows show the gene name, GenBank Accession No., protein localization, 10 tumor-to-OSE ratios that were observed in the HDAH (only ratios above 2.5; normal-to-OSE ratios are omitted for they lied all below 2.5), followed by 22 tissue-to-OSE ratios determined in the RT-PCR (for clarity, only ratios above 1 are displayed). The columns are duplicated for *14.3.3.* and *HE4* because two clones were selected for them by HDAH. *Putative Progesterone binding protein*(ProgBP): progesterone binding proteins can be found in low-grade breast cancers and in some ovarian cancer cell lines. The homologous rat sequence has a transmembrane region (Falkenstein et al., 1996), indicating that our clone might also be membrane-bound. *Ryudocan* (abreviated as Ryu.) is a cell-surface proteoglycan with a transmembrane domain; it is expressed in an extensive array of human tissues (Kojima et al., 1993). *HE4* is an epidermal, epididymis-specific protease inhibitor that is thought to be involved in the maturation of spermatozoa (Kirchhoff et al., 1991). The putative *HE4* protein has a leader sequence and it is speculated that it is secreted. *Mucin1* (Dong et al., 1997) is expressed on the cell surface of non-mucinous ovarian tumors with either low malignant or invasive potential. *14.3.3* codes for a cytosolic protein kinase regulator protein that shows elevated expression levels in lung cancer tissues (Nakanishi et al., 1997). *BA46*, also known as lactadherin, is a cell-surface protein expressed in human breast carcinomas. It has been used successfully as a target for experimental breast cancer radioimmunotherapy (Couto et al., 1996). *Beta actin* is a cytoskeletal protein with differential expression in acute myelolytic leukemia (Blomberg et al., 1987) and high expression in colorectal carcinomas (Naylor et al., 1992). *E16* codes for an integral membrane protein that was isolated from peripheral blood lymphocytes (Gaugitsch et al., 1992). It is expressed in colorectal and other human carcinomas (Wolf et al., 1996).

E16 shows tumor-to-OSE ratios above 2.5 in three tumors (with a mean of 5.3 compared to a mean of 4.4 in the HDAH). It also shows high ratios for two normal ovaries and placenta. The low expression in PBL and the high average ratios for the tumors make it a possible marker candidate.

HE4 shows a clear tumor-restricted expression, making its pattern resemble that in the HDAH. Most importantly, the results suggest that it is not expressed in peripheral blood lymphocytes. As noted in the beginning of this section, this accordingly represents a candidate for a serum marker assay. The difference in the mean rates of overexpression measured by RT-PCR (11 ×) and HDAH (4.1 ×) can be attributed either to

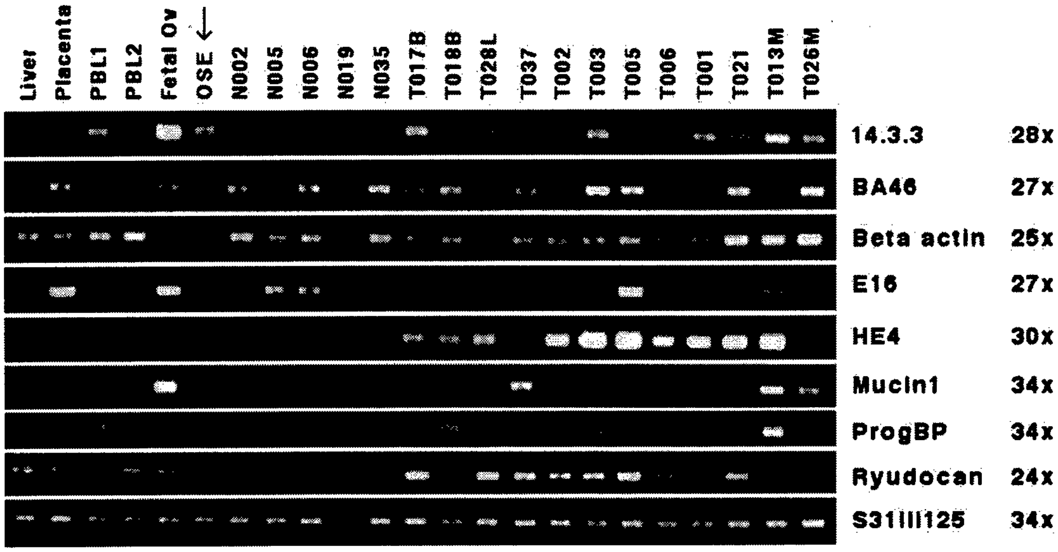

Fig. 3. Expression monitoring by RT-PCR. Eight genes (plus one control) are tested in 23 tissues. Tissue names are on top; OSE is marked with an arrow. Tissues starting with an N are normal ovaries, and those starting with a T are ovarian tumors. The *S31iii125* gene serves as a control. The number of PCR cycles is indicated behind each gene name (on the right). ProgBP stands for 'putative progesterone binding protein'. The PCR bands are in the range of 420–660 bp. All reactions had been performed in four parallel sets with one set shown here.

the better signal-to-noise ratio in the RT-PCR or to the different tumor samples used.

Mucin 1 shows a high RT-PCR value in fetal ovaries, suggesting that this might be a fetal gene that is re-expressed in the tumor. It shows strong bands in three out of the 12 tumors, two of them metastatic and one an early stage tumor, resulting in a mean tumor-to-OSE ratio of 3.0. This result correlates with that of the array hybridization.

The *Putative progesterone binding protein* shows a high tumor-to-OSE ratio for only two tumors, one being similar in stage to a tumor used in the HDAH. All other tumors show medium high ratios but so do the normal tissues, including the PBL. The strong expression in the metastasizing tumor may indicate a role as a marker for tumor staging, prognosis or stratification.

The transcript of *ryudocan* displays a similar pattern of expression as *HE4*, and the mean the tumor-to-OSE ratio of 4.3 are is slightly higher than the one determined by HDAH (where it was 6). The presence of *ryudocan* mRNA in liver, PBL and placenta means that the protein might normally be found in the blood, thus making it a less suitable marker candidate.

3.5. *Confirmation of overexpression of HE4 by Northern blot analysis*

Of the eight genes tested in the RT-PCR, only *HE4* shows a clear tumor-restricted expression pattern. To further confirm the cancer-restricted expression of *HE4*, we used a Northern blot (Northern Territory®, Invitrogen, San Diego, CA) that contained total RNA from ovaries from four patients who had unilateral ovarian cancer. RNA from both the affected and the unaffected ovary was present on the blot (loaded adjacent to each other). Fig. 4 shows that *HE4* is expressed in two ovarian carcinomas but not in the matching normal ovaries. *HE4* cannot be detected in the tumors nor in the normal ovaries of two other patients. The ratios of *HE4* expression between the unaffected and

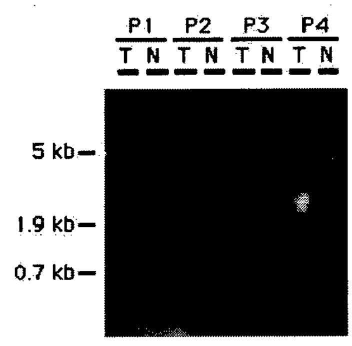

Fig. 4. Northern hybridization of *HE4*. The Northern blot contains RNA from ovarian tumor and matching non-affected ovary from four patients. *HE4* is expressed in two tumors but not in the normal tissue of the same patient. A Digoxigenin-labeled riboprobe was prepared from a 500 bp *HE4* PCR product cloned in a vector. The probe was hybridized over night at 68°C and washed in 2× SSC, 0.1% SDS for 15 min at room temperature; 2× SSC, 0.1% SDS for 20 min at 68°C; 0.1× SSC, 0.1% SDS for 2×15 min at 68°C. The hybridized RNA was visualized using the DIG detection kit (Boehringer Mannheim). The membrane was exposed to X-ray film for 15 min.

the affected ovary was 6.1 for patient 1 and 4.5 for patient 2. Thus, *HE4* is also a candidate for a tumor-staging, prognosis or stratification marker.

3.6. Conclusion

From the 21 500 clones, we chose 43 that were overexpressed in ovarian tumors by HDAH with homology to characterized genes. We chose eight genes for expression validation by RT-PCR. From these eight, seven genes displayed tumor-to-OSE ratios similar to those measured in the HDAH, albeit with different tumor tissues matching grade and stage. Seven of these eight display expression in normal tissues; only *HE4* showed a clear tumor-restricted expression pattern. We conclude that the *HE4* message is significantly overexpressed in a variety of ovarian tumors relative to normal tissues or OSE, thus making it a potential candidate for a marker protein.

The results support the validity of using HDAH combined with a second quantitation method for the identification of genes that are overexpressed in cancers as compared to normal tissues. We are preparing an antibody against *HE4* to further analyze whether it indeed could be a diagnostic marker for ovarian cancer.

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